Light Controlled Bio-inspired Small Molecules

by

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Abstract

The work presented in this thesis examines the design, preparation and evaluation of light controlled biologically inspired small molecules. Incorporation of light sensitivity was achieved through the introduction of a photoresponsive diarylethene group, into a specific position within the molecular structure of a biologically relevant compound. The diarylethene class of photoresponsive molecules can absorb light of a specific wavelength, and subsequently undergo a reversible, light induced isomerization reaction, to generate a new structure with a unique set of chemical and physical properties. This photoisomerization process, also referred to as photoswitching, allows for reversible manipulation of the modified biomolecule’s properties, and consequently its ability to interact with a target using light. During this thesis, two examples utilizing the diarylethene framework are presented as a means to control the properties; either geometric (steric) or electronic, of photoresponsive small molecules using light energy.

In the first example, featured in Chapter 2, light is used to alter the inhibitory activity of a photoswitchable enzyme inhibitor. The design relies on the structural and geometrical changes that accompany photoswitching of the central diarylethene to achieve this. A series of inhibitor candidates, based on the bisindolylmaleimide class of protein kinase inhibitors, were synthesized and investigated. A key derivative exhibited good \textit{in vitro} inhibition, and its inhibitory activity could be switched from an inactive, “off” state, to an active, “on” state, with brief exposure to non-damaging visible light.

In the second example, featured in Chapter 3, light is used to control the electronic properties of a photoisomerizable Pyridoxal 5'-phosphate (PLP) mimic and influence the rate of a racemization reaction. The design combines the essential structural features of PLP, which are an aldehyde and a pyridinium, with a diarylethene photoswitch. The inherent changes that take place to the structure of the diarylethene with photoisomerization, effectively allowed for reversible modulation of the degree of electronic connection between the aldehyde and pyridinium. Consequently, control over the cofactor mimic’s reactivity towards substrate was possible.
Keywords: Photoswitching, Diarylenes, Protein Kinase C, Pyridoxal Phosphate, Light controlled enzyme inhibitor, Light controlled catalysis
Dedication

To my Grandmother, P. M. Wilson.
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Figure 3.7 – Overlap of the $^1$H NMR spectrum from 12.0-6.5 ppm of the ring-open isomer 3.1-o (bottom) and the photostationary state mixture (top) containing 97% of 3.1-c (and the remaining 3% assigned to 3.1-o).

Figure 3.8 – Selected region of the $^1$H NMR spectrum (CD$_3$CO$_2$D/D$_2$O (9:1), 400 MHz) of the photostationary state mixture obtained from irradiation of 3.1-o with (365 nm light for 30 minutes). Using the ratio of the integrals from the aldehyde peak of 3.1-c and 3.1-o the photostationary state is estimated to be 97%.
Figure 3.9 – Proposed mechanism for racemization and proton-deuterium exchange catalyzed by the active ring-open isomer 3.1-c. The amino group of alanine reacts with the aldehyde to generate the corresponding aldimine product. Deprotonation gives the quinoidal intermediate. Reprotonation of the C=N double bond regenerates the D/L aldimine, then hydrolysis in the presence of D_{2}O gives alanine as a racemic mixture.

Figure 3.10 – Partial 1H NMR spectrum (CD_{3}CO_{2}D/D_{2}O 9:1) for 3.1-c (a) and 3.1-o (b) at t = 0, t = 140 h and t = 20 days. The purple dotted line is drawn to highlight the maximum intensity of the alanine quartet.

Figure 3.11 (a) Proton-deuterium exchange experiment using L-alanine in the presence of either the ring-open isomer 3.1-o or the ring-closed isomer 3.1-c. (b) Plot of the percentage of deuterium exchange versus time. (c) Changes in percentage deuterium exchange over time in response to alternating cycles of irradiation with UV and visible light.

Figure 3.12 - Partial 1H NMR spectra (400 MHz, D_{2}O) of (a) the alanine (5.7 × 10^{-2} M) isolated after reaction with 3.1-c performed in CD_{3}CO_{2}D/D_{2}O (9:1 v/v) (bottom), and the addition of 0.1 molar equivalent of the sodium [(R)-1,2-diaminopropane-N,N,N',N'-tetraacetato]samarate (III) (top). (b) optically pure L-alanine (7.5 × 10^{-2} M) (bottom), the same solution with the addition of 0.1 molar equivalent of the sodium [(R)-1,2-diaminopropane-N,N,N',N'-tetraacetato]samarate (III) (middle), and the addition of a drop of a similar D-alanine solution (7.8 × 10^{-2} M) (bottom) and (c) optically pure D-alanine (7.8 × 10^{-2} M) (bottom), the same solution with the addition of 0.1 molar equivalents of the sodium [(R)-1,2-diaminopropane-N,N,N',N'-tetraacetato]samarate (III) (middle), and the addition of a drop of a similar solution of L-alanine (7.5 × 10^{-2} M) (bottom). All samples were basified to pH 10–11 with NaOD (40% w/w in D_{2}O) to ensure binding to the chiral shift reagent. The inconsistencies in the chemical shifts between samples is due to the high pH sensitivity of the coordination to the chiral shift reagent.
### List of Acronyms

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<tr>
<td>&gt;</td>
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<td>approximately</td>
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<td>HEPES</td>
<td>(4-(2-hydroxyethyl)-1-piperazine)ethanesulfonic acid</td>
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NCS  n-chlorosuccinimide
ng  nanograms
N.M. not measured
nm  nanometers
nM  nanomolar
NMR  nuclear magnetic resonance
O.D.  optical density
p  para
PDB ID  Protein Data Bank ID
Pd(PPh₃)₄  tetrakis(triphenylphosphine) palladium (0)
Ph  phenyl
pH  -log (hydrogen ion concentration)
PKC  protein kinase c
PLP  pyridoxal 5’-phosphate
pM  picomolar
PMA  Phorbol 12-myristate-acetate
ppm  parts per million
PS  pseudosubstrate
PSS  photostationary state
Rf  retention factor
rpm  revolutions per minute
RT  room temperature
s  singlet
s  seconds
SAR  structure activity relationship
sec  second
SOCl₂  thionyl chloride
Sn  tin
t  triplet
t  time
t-BuLi  tert-butyllithium
TBAB  tetrabutylammonium bromide
TEA  triethylamine
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<tr>
<td>ν&lt;sub&gt;e&lt;/sub&gt;</td>
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1 Introduction

1.1 Light as a stimulus for biochemical transformations

Light can be precisely tuned and administered with a high degree of spatial and temporal resolution.\(^1\) With modern optics, the wavelength of light, power density and exposure settings can all be selectively programmed to create a custom output profile that is suitable for non-invasively controlling dynamic processes in a biological context.\(^2\) Light provides several exciting advantages in comparison to traditional methods, for example with chemical effectors or through genetic manipulation. These techniques often suffer from a common set of limitations.\(^3\) Chemical agents give the user little control over their activity profile once administered. Genetic mutation techniques take place on a relatively slow timescale; where the cell has time to compensate for the effects by other means.\(^4\) Whereas, with light stimuli, a rapid change can be induced in a specific location and at a desired time point.

The unique features offered with light energy can be harnessed for use in biological systems that are traditionally non light-responsive. This can be achieved through incorporation of a light sensitive chromophore, such as a molecular photoswitch, into the biomolecule of interest.\(^5\) Briefly, this approach relies on the changes that occur within the structure and/or properties of the chromophore when it absorbs light of different wavelengths.\(^6\) Researchers have applied this technology to selectively control drug release,\(^7\) modulate protein-protein interactions,\(^8\) control neuronal polarization,\(^9\) activate transcription,\(^10\) perturb protein and peptide structure\(^11\) and influence enzyme catalysis.\(^12\) Ultimately, providing a powerful tool for studying cellular dynamics, deconvoluting the roles of biomolecules and even as a therapeutic treatment.
Despite the elegant examples designed to date, that illustrate the success of this concept, harnessing light energy to control a biological function is a challenging process. Unfortunately, there is a lack of general or straightforward procedures currently available to introduce light sensitivity into biological molecules, so that precise changes to their properties can be made. As was mentioned in the previous paragraph, optical control over biological function, is frequently accomplished by attaching a light sensitive chromophore (either synthetically or naturally derived) directly to a biomolecule (i.e. a protein) of interest.\textsuperscript{13} However, two general drawbacks and challenges, that are frequently encountered include:\textsuperscript{14}

1) Selecting an appropriate attachment site to place the light sensitive chromophore. The activity before and after light exposure is difficult to predict. Simply incorporating a light responsive group does not guarantee successful optical control. Therefore, identification of an optimal attachment site frequently involves careful design, as well as trial and error. The chromophore should be incorporated in such a way that a large enough structural or functional change is accomplished. If these are too slight, than the changes that accompany light exposure can often be absorbed by the biomolecule - consequently no effect is achieved. On the other hand, there is also the possibility that incorporation of chromophore disrupts the biomolecule structure too extensively and hinders its function altogether.

2) Selecting a suitable incorporation method. The attachment and isolation conditions must be compatible with the biological target. Delicate biomolecules may not be able to tolerate common chemical manipulation methods. This will depend on the type of chromophore used and the ease with which the biomolecule can be derivatized.

The difficulties in engineering and administering light responsive biological molecules, hinders the extension of optical control techniques to most biological processes. Therefore, ways to expand and improve this technology are required in order to increase its applicability to a wider range of systems.

An alternative approach that could avoid some of the problems outlined above, is to engineer photoisomerizable small molecules that can change their ability to interact with a particular biological target. For example, the introduction of a molecular photoswitch into the structure of existing effector compounds, (i.e. inhibitors, activators
and cofactors), could prove to be a fundamentally simpler technique to render a biological transformation light responsive. A modified effector molecule could then be used directly with native biomolecules. Thus, eliminating many of the challenges faced during the design of light controlled macromolecules (i.e. proteins), including selection of a chromophore attachment site and incorporation method. This topic is expanded on in Section 1.4.

The concept of using small photoresponsive biomolecules is not new.\(^\text{14}\) However, relatively few examples have been developed to date, that meet all of the requirements for this technology to become a feasible study tool. First, the photoresponsive small molecule must show a high affinity, that is at least comparable with the existing analogue that inspired the design. Second, the difference in activity between the inactive and active isomers should be sufficiently large to allow for ‘on-off’ switching of their activity under physiological conditions. Third, the light induced property changes should be rapid, efficient, and utilize low energy light to trigger isomerization, so that prolonged irradiation of biological systems with potentially harmful light is avoided. Few systems meet this list of criteria. Therefore, further investigation is needed in order to understand how photoresponsive small molecules can be appropriately designed to meet these demands, and to expand their use as a practical way to gain control over a biological process with light.

This thesis reports investigations on how the geometric and electronic changes that occur to light controlled photoresponsive small molecules can be exploited to influence the activity of two biologically inspired processes. The first example, featured in Chapter 2, uses the changes in geometry that accompany photoisomerization of a photoresponsive diarylethene inhibitor to influence the activity of a protein kinase target. The second example, featured in Chapter 3, uses the changes in the electronic properties that take place to the diarylethene core with light to influence the cofactor’s ability to convert substrate to product.
1.2 Introduction to Photochromism and Molecular Photoswitches

1.2.1 Definition of photochromism

Photochromism, (or photoswitching), is defined as a reversible light induced transformation of a molecule between two isomers having different absorption spectra.\textsuperscript{15} There are different types of organic based photochromic reactions, for example, involving isomerization of double bonds, pericyclic rearrangements, intramolecular proton transfer, intramolecular group transfers, dissociation processes and electron transfers.\textsuperscript{16} Each of these have different mechanisms by which photochromism occurs, however provided that the reversibility criterion is met (that is they possess the ability to reversibly interconvert between two or more compounds having distinct absorption spectra) they are classified as photochromic.

The light induced photoconversion that occurs within photochromic systems can be represented by the cartoon in Scheme 1.1. One isomer, termed species ‘A’ absorbs light of wavelength, $\lambda_1$, and undergoes conversion to isomer ‘B’. Depending on the structure, reversion to isomer ‘A’ can be achieved with light of $\lambda_2$ or can occur spontaneously, denoted as $\Delta$.

![Scheme 1.1](image)

Scheme 1.1 – Cartoon illustration of a photochromic reaction. Isomer ‘A’ absorbs light of wavelength, $\lambda_1$ and subsequently undergoes photoisomerization to generate isomer ‘B’. The reverse process can be achieved by irradiation of isomer ‘B’ with another wavelength of light, $\lambda_2$ or depending on the structure of the photochromic molecule and the surrounding temperature, the reversion process could also occur spontaneously in the dark.
1.2.2 Selected examples of synthetically derived organic photoswitchable molecules

Organic based pericyclic reactions, which include the diarylethene class of molecules are the focus of this thesis and will be overviewed in more detail in the next section (Section 1.3 – Diarylenes). However, a brief introduction to some commonly encountered photoswitchable molecules are included in this section for comparison.

Photochromism can be generally classified into two different types, depending on the thermal stability of the isomeric forms, these are typically referred to as T-type and P-type.\(^\text{15}\) In T-type (or thermally reversible type) photoswitchable molecules, the photogenerated isomer(s) are unstable and revert spontaneously to their initial state in the dark, the majority of photoswitches belong to this type, for example azobenzenes, spiropyrans, spirooxazines and hemithioindigos.\(^\text{15}\) A few examples of T-type photoswitchable molecules are given in Scheme 1.2 below.

### Scheme 1.2 – Examples of synthetically derived photoswitchable molecules that exhibit T-type photochromism

- **azobenzene**
  - \[ R\text{N} \quad \text{trans} \quad \text{hv}_1 \quad R\text{N} \quad \text{cis} \]
  - \[ R \quad \text{hv}_2 \text{ or } \Delta \]

- **hemithioindigo**
  - \[ R\text{S} \quad \text{trans} \quad \text{hv}_1 \quad R\text{S} \quad \text{cis} \]
  - \[ R \quad \text{hv}_2 \text{ or } \Delta \]

- **spiropyran**
  - \[ R\text{S} \quad \text{spiropyran} \quad \text{hv}_1 \quad R\text{S} \quad \text{merocyanine} \]
  - \[ R \quad \text{hv}_2 \text{ or } \Delta \]
The majority of examples published to date, that have used synthetically derived photoswitchable molecules to control various features of biological molecules, fall into the T-type category. With azobenzenes being by far, the most commonly encountered choice. Azobenzenes undergo a cis/trans isomerization. The trans isomer is typically 10-12 kcal/mol more stable than the cis isomer, so that in the dark and at equilibrium, the trans conformation is the dominant (>99%) isomer present. The cis isomer is typically produced with UV irradiation, in yields that vary with substituent choice, however with most derivatives do not exceed photoisomerization yields of 80%. The trans isomer can be regenerated by either thermal relaxation from the cis state in the dark or through irradiation with visible light (~450 nm is typical). In contrast to the nearly planar trans isomer, the cis isomer adopts a bent conformation with one of the phenyl rings twisted ~55° out of plane. In addition to the shape change that occurs with photoisomerization, the length of the molecule changes by at least 3.5 Å.

Hemithioindigos consist of a thioindigo fragment connected to a stilbene fragment via a central double bond. The double bond, like azobenzene can be isomerized between the cis and trans forms using light. Their absorption is red shifted, allowing for visible light induced photoisomerization in both directions. The cis configuration is more thermodynamically stable than the trans isomer. The barrier for trans to cis isomerization is >27 kcal/mol. A value that is usually higher than the barrier for the thermal cis to trans isomerization for most azobenzenes.

Spiropyran is the second most frequently encountered photoswitch used in biological applications, likely because of the pronounced change in geometry and polarity that take place with photoisomerization. The difference in polarity is predicted to change from 2-5 D for spiropyran to 20 D for merocyanine. UV light irradiation converts the bulkier and typically colourless spiropyran isomer to the planar, coloured merocyanine isomer. The reverse process of ring-closure proceeds thermally at relatively slow rates (although this is solvent dependent) or through irradiation with visible light. Spiropyran isomerization behaviour and stability are often difficult to predict. Typically, the spiropyran isomer predominates, however, the merocyanine isomer can predominate in polar solvents when polar substituents are included in the molecular structure.
The second type of photochromism includes molecules which are thermally irreversible or P-type (photochemically reversible). These include diarylenes and furylfulgides. Derivatives that fall into this class of photoswitchable molecules, often do not revert to their initial state even at elevated temperature, >100 °C (although this is substituent dependent). Both diarylenes and fulgides contain a 1,3,5-hexatriene in their structure and with light absorption, can undergo an electrocyclization reaction from the ring-open isomer to the ring-closed isomer. The isomerization process is often accompanied by a pronounced change in electronic structure and geometry.

Scheme 1.3 – Examples of synthetically derived photoswitchable molecules that exhibit P-type photochromism.

Depending on the application, one type of photoswitching may be more desirable than another. P-type photochromism allows the user to have full control over the isomeric ratio, as the interconversion between the two isomers can be achieved only with light. However, with T-type photoswitchable molecules, the spontaneous reversion from the meta-stable isomer to the original state, and light can still be used to adjust the ratio of the two species, simply by adjusting the illumination time and intensity.

1.3 Diarylenes

The diarylene class of photoresponsive molecules was introduced in the previous section, however a more detailed description of their structure, photochemical
behaviour, and an overview of how light can be used to influence the physical and electronic properties will be included here.

1.3.1 Introduction and basic structure

The core structure of the diarylethene class of photoresponsive molecules are made up of 1,3,5-hexatriene system, highlighted in bold in Figure 1.1 below. The molecule contains a central alkene with two pendent heteroaryl groups connected in the 3,3’ positions (see Figure 1.1, pink lettering, for carbon atom numbering). The heteroaryl groups are amenable to modification, while a 5-membered ring (as shown in the figure below) is typical, (i.e. thiophene, oxazole, furan, thiazole), a fused ring system can be also be used as well, (i.e. benzothiophene, benzofuran, indole and phosphole).22 Furthermore, the substituents on the rings, labelled R₁-R₆ can all be tuned to alter the optical and chemical behaviour of both photoisomers to fit a desired application.20,23

The central alkene is often cyclic (labelled cyclic ring system in Figure 1.1), for example a cyclopentene, hexafluorocyclopentene, maleimide or anhydride etc. to prevent rotation of the central alkene bond from a cis to trans configuration. The 2,2’ internal positions are frequently substituted with alkyl or aryl groups to enhance reversibility, and prevent irreversible oxidation and aromatization of the ring-closed structure.20

![Figure 1.1 – Diarylethene basic structure and substitution patterns.](image-url)
The ring-open isomer of the diarylethene, DAE-o can absorb light of a specific wavelength, (often UV light, but not always) and undergo an intramolecular 6-π electrocyclization reaction to produce a 1,3-hexadiene scaffold, denoted as DAE-c. The reaction generates a mixture of two enantiomers, the (R,R) and (S,S) species, as the bond hybridization of the aryl groups change from sp\(^2\) to sp\(^3\) after formation of a new \(\sigma\)-bond between carbons 2,2'.

\[
\begin{align*}
\text{DAE-o} & \quad \xrightleftharpoons[\text{visible or \(\Delta\)}]{\text{UV}} \quad \text{DAE-c mixture of} \\
& \quad \begin{array}{c}
\text{(R,R)} \\
\text{(S,S)}
\end{array}
\end{align*}
\]

Scheme 1.4 – Diarylenes (DAEs) can be reversibly interconverted between the ring-open isomer, DAE-o and the ring-closed isomer DAE-c using UV light for the ring-closure process. Visible light (or heat) can be applied to induce the ring-opening process. The 6-π electrocyclization generates two diastereomeric products, (R,R) and (S,S).

The isomerization reaction is accompanied by a change in the molecule’s absorption spectrum, and often, a visible colour change can be detected as the ring-closed isomer DAE-c has an extended π-conjugated system, shifting its absorption into the visible region. The cyclization process can be reversed, to regenerate the original ring-open isomer DAE-o, by exposure to another wavelength of light, typically in the visible region. An example of the light induced changes in the absorption spectrum of a diarylethene is given in Figure 1.2.
Figure 1.2 - An example of the changes to the absorption spectrum when the ring-open isomer, \textbf{DAE-o} is irradiated with light to produce \textbf{DAE-c}. A colour change from yellow to purple accompanies the isomerization process for this particular example.

When the yellow coloured ring-open isomer is irradiated with light, it undergoes cyclization to produce the purple coloured ring-closed isomer. The isomerization is accompanied by a dramatic change in the absorption spectrum. The band from the ring-open isomer, centered at \textasciitilde450 nm, decreases in intensity along with the appearance of a new, red shifted band at 570 nm, and a second set of bands at \textasciitilde410 nm. The process is reversible, and can be initiated by irradiation with light of wavelength >500 nm, where the ring-closed isomer absorbs.

Generally, the ring-closed isomer is considered to be thermally stable, with little cycloreversion, even at elevated temperatures. However, spontaneous reversion in the dark can occur. The rates for spontaneous reversion are both structure and temperature dependent. In addition to the changes that occur in the structure and absorption spectrum, a number of other properties can be reversibly modulated as well, including geometry, electronic structure, refractive index and fluorescence emission.\textsuperscript{20} A few examples of how changes in the \textbf{DAE} geometry and electronic structure with light are featured later on in this section.
1.3.2 Photochemical ring-closure

The photochemical ring-closure process is dependent on the arrangement of the π-orbitals of the 1,3,5-hexatriene. In most instances, the ring-open isomer exists as an equilibrium mixture of two distinct conformers in solution,\textsuperscript{20,25,26} see Scheme 1.5 below. In the “parallel” conformer (\textit{DTE-o parallel} in Scheme 1.5), the two pendent aryl groups face the same direction and have a mirror plane of symmetry. Whereas in the “antiparallel” conformer (\textit{DTE-o antiparallel} in Scheme 1.5), the two aryl groups face the opposite directions and exhibit an axis of $C_2$ symmetry. Due to conservation of orbital symmetry, described by the Woodward-Hoffman rules, the photochemically induced ring-closure process to produce \textit{DTE-c} can only occur from a conrotatory electrocyclization of the antiparallel conformer.\textsuperscript{27}

\begin{center}
\begin{tabular}{c}
\includegraphics[width=\textwidth]{image.png}
\end{tabular}
\end{center}

\textbf{Scheme 1.5 –} The ring-open isomer of diarylenes, \textit{DTE-o} can exist as either the \textit{parallel} conformer which is not photoactive, or the \textit{antiparallel} conformer. Only the latter can undergo light induced electrocyclization to give the ring-closed isomer \textit{DTE-c}.

In the DAE class of photoresponsive molecules, the photoisomerization reaction occurs from the lowest unoccupied molecular orbital, or the LUMO energy level, $\psi_4$ see Figure 1.3 (a) for the orbital arrangements of $\psi_1$-$\psi_6$ possible for a typical 1,3,5-hexatriene system.\textsuperscript{28} Upon inspection of the orbital phases, sufficient orbital overlap between the terminal C$_1$ and C$_6$ carbon atoms can be achieved by a conrotatory motion of the π-orbitals from the LUMO. Rotation of the orbitals in the LUMO in the same direction (conrotatory) either left or right, generating new sigma bond, Figure 1.3 (b) and
yields the 1,3-cyclohexadiene. Whereas with a disrotatory motion from the LUMO state, the phases of the C₁ and C₆ π-orbitals are opposite, and consequently a new sigma bonding orbital is not created. Alternatively, from the ground state, HOMO energy level, a thermal disrotatory cyclization is possible and can generate a new sigma bond.²⁷,²⁸ See Figure 1.3 (c) below.

**Figure 1.3** – (a) Orbital configuration diagram for a typical 1,3,5-hexatriene system. (b) possible modes of conrotatory electrocyclization from the LUMO or excited state energy level or (c) possible modes of disrotatory electrocyclization from the HOMO or ground state energy level generates sufficient overlap of the π-orbitals forming a new σ-bond.²⁸

The electrocyclization reaction is highly stereospecific, generating a set of enantiomers for the photochemical process and alternatively a different set of products are possible from the ground state process. In the conrotatory mode, the two internal
groups labelled B and C in Figure 1.3 (b) are trans to each other, the external groups A and D are trans to one another. Whereas in the disrotatory mode Figure 1.3 (c), the internal groups B and C are cis to one another, and the external groups A and D are also cis to one another. The thermally induced cyclization from the disrotatory mode does not generally occur due to the high energy barrier required to overcome the steric bulk around the 2,2'-internal positions, and create sufficient orbital overlap for thermal ring-closure.

1.3.3 Photostationary state

During the photoinduced conversion of the ring-open to ring-closed isomer, an equilibrium is eventually established where the changes in concentration of the ring-closed and ring-open isomer begin to slow, and eventually stop, with subsequent light irradiation. This point is referred to as the photostationary state (PSS). The photostationary state represents the relative yield of the photocyclization reaction under a particular set of conditions, (wavelength, solvent, temperature etc.) see equation below:

\[
PSS = \frac{\# \text{ of isomerized molecules}}{\text{total } \# \text{ of molecules}}
\]

Diarylethenes are typically described as exhibiting high photoconversion yields, with the PSS nearly quantitative. However, the PSS and the stability of the ring-closed isomer are dependent on the molecule’s structure, and in particular the types of heteroaryl groups appended to the central alkene. Ring-closure yields can vary substantially, from excellent to negligible with substituent selection. The substituents on the internal positions can also have an influence on the conversion yield. When the aryl groups are thiophene, thiazole, furan, which have lower aromatic stabilization energies, the resulting ring-closed isomers tend to be stable and exhibit relatively good conversion yields. However, when the aryl groups are replaced with pyrrole, phenyl, or indole,
which have higher aromatic stabilization energies, the PSS and stability of the ring-closed isomers are usually moderate to poor.

Additionally, the photostationary state is dependent on the irradiation conditions, including the intensity of the light source, the wavelength range, solvent, concentration of the solution and temperature.

The ring-closure process of diarylethenes can often be conveniently monitored using UV-visible absorption spectroscopy, by examining the changes that occur within the spectrum with light irradiation. An example of a UV-visible absorption spectrum of a solution of a photoresponsive diarylethene derivative is given in Figure 1.4. When the ring-open isomer, DAE-o is irradiated with light, the structure undergoes cyclization to produce the ring-closed isomer DAE-c. Figure 1.4 (a) shows the sequential changes that occur with photocyclization over a 10 minute time period. As the ring-open isomer is generated, a new peak appears in the visible region at ~575 nm, and several peaks in the UV region from ~350-400 nm increase in intensity. As the product, DAE-c accumulates, the conversion rate slows, and eventually reaches the photostationary state. At this point, there are no further changes within the spectrum with continued irradiation, represented by the pink dotted line in Figure 1.4 (a). The UV-visible absorption spectra of the pure ring-closed isomer DAE-c, the photostationary state mixture DAE-PSS and ring-open isomer DAE-o are provided for comparison Figure 1.4 (b). This reveals that the isomerization process is not quantitative, rather under these conditions, ~42% conversion is possible.
Figure 1.4 – (a) Changes in the absorption spectrum of a solution of the ring-open isomer, DAE-o as it is irradiated with light to generate ring-closed isomer, DAE-c. The solution was exposed to light at 60 second intervals, and the absorption measured after each interval. When no further changes in the absorption occur, the photostationary state is reached. (b) Comparison of the absorption spectrum of the ring-open isomer DAE-o, ring-closed isomer DAE-c, and the photostationary state mixture, DAE-PSS.

1.3.4 Modulation of diarylethene geometrical configuration with light

In addition to the optical properties, a change in the structure and 3-D geometry accompanies the isomerization of the diarylethene photoswitch. The ring-open isomers of diarylethenes are generally flexible, and two aryl substituents are free to rotate about the central double bond, see Scheme 1.6. Therefore DTE-o can adopt a range of conformations, which in solution, usually exist as an equilibrium mixture.\textsuperscript{20,23} The orientation of the aryl groups influence the overall 3-D geometrical configuration of the photoswitch. The distance between the aryl groups, and the dihedral angle they make with the central alkene can all be reversibly modified. Whereas in the ring-closed isomer, DTE-c, the $\sigma$-bond connecting the 2,2'-positions of the aryl groups, hinder its rotation ability, creating a more planar and rigid system.
Scheme 1.6 - Changes that occur to the DTE geometry with light exposure. The thiophene rings of the ring-open isomer, DTE-o, are free to rotate and therefore a number of different orientations are possible in this isomer. These typically exist as an equilibrium mixture. Whereas the ring-closed isomer, DTE-c, the new σ-bond formed at the 2,2’-position, restricts free rotation, creating a rigid and planar system.

The geometric changes that occur with photoswitching have been exploited to influence the behaviour of the DTE system. This feature has been useful in a range of applications, including controlling the binding affinity to protein targets, self-assembly into supramolecular structures, catalytic function, and reactivity. A few examples are given below to illustrate how the different geometric changes that occur with photoswitching are harnessed for control over different features using light.

**Light controlled distance between pendant binding sites of an enzyme inhibitor:**

This example demonstrates how the diarylethene architecture allows for the distance between the two binding sites of a bidentate inhibitor to be reversibly controlled.\(^{29}\) In the ring-open isomer, the distance between the pendant copper imino acetate and the sulfonamide groups was estimated by the authors to be 7-10 Å, which matches the appropriate distance between the two binding sites on the enzyme. The ring-closed isomer, on the other hand, has an estimated distance of ~11 Å between the two pendant sites. Its more rigid structure, and larger span between the two ends of the ring-closed isomer, prevents the simultaneous binding to both sites. Therefore, the ring-open exhibits higher inhibitory activity than the ring-closed isomer.
Scheme 1.7 – In the flexible ring-open isomer, the inhibitor possesses the appropriate distance between the pendent sulfonamide and copper imino acetate groups to allow for both of them to interact simultaneously with enzyme's two binding sites. In contrast, the larger distance between the binding groups in the ring-closed prevents this.29

The authors report an approximately 60-fold change in activity using light. As predicted, the ring-open isomer allowed for a larger extent of inhibition with a $K_i = 5$ nM for Human carbonic anhydrase. In contrast, the ring-closed had a measured $K_i$ of 300 nM under the same conditions.29

**Light controlled antimicrobial peptidomimetic:**

Recently, Komarov et al, introduced a photoresponsive dithienylethene group into a cyclic peptide antimicrobial to control its ring size, geometry, and hydrophobicity.30 Molecular simulations predicted that the more flexible ring-open isomer would be able to adopt a conformation that resembles the antimicrobial peptide, gramicidin S. This was expected to translate into a higher activity in comparison to the ring-closed isomer.
Scheme 1.8 – Light induced changes in the ring size and amphiphilic nature of the diarylethenyl based cyclic peptide allow for effective control over bacterial growth.30

The site of attachment of the dithienylethene was varied and the authors demonstrate approximately 16-fold change in antimicrobial activity with photoisomerization, allowing for effective control over the growth of several bacterial strains.30 As predicted, the ring-open isomer, was distinctly more effective at inhibiting bacterial growth than the ring-closed form, in all of the microorganisms tested during the study. They concluded that the ring-open isomer, although less hydrophobic than gramicidin S, is more hydrophobic than the ring-closed isomer. The difference in antimicrobial activity could be attributable to changes in surface charge distribution of the molecule, that occur as a result of photoswitching.

**Light controlled structural architecture of a palladium-DTE complex:**

In the example below, the authors demonstrate how light can be used as a trigger to modify the structural architecture of a Pd-DTE complex by varying the distance between the metal-binding donor ligands.31 See Scheme 1.9, for the predicted structure of the ligand, and resulting Pd complexes formed in the presence of the ring-open and ring-closed isomers. The ligand is made of a central DTE with two para-substituted
pyridines appended to the external positions on the thiophene rings. The DTE functions as a bridge, to change the binding angle between the pyridine ligands and the metal and influence the possible dimensions of the complex.

Scheme 1.9 – The diarylethene geometry controls the supramolecular architecture of the resulting metal complex. The parallel conformation of the ring-open isomer, with a smaller distance between the ligand donor groups, assembled into an ~2.2 nm triangular complex. Irradiation of the complex containing the ring-open isomer produces the planar ring-closed isomer, which assembles into a larger spherical ~6.4 nm complex. (Reprinted with permission from John Wiley & Sons, Inc).31

A mixture of the ring-open isomer with a Pd salt, in a 2:1 ratio generates a small ‘triangular’ shaped ring with the stoichiometry Pd₃L₆ (where L = DTE ligand). Irradiation
of the resulting complex with 313 nm light generates the ring-closed isomer, and this leads to dissociation of the triangular structure. Due to the molecule’s planar, rigid structure, it reassembles into a large spherical structure with the stoichiometry, Pd$_{24}$L$_{48}$.$^{31}$

**Light controlled stereoselectivity of a reaction:**

In the example outlined in Scheme 1.10, the authors demonstrate that the stereochemical selectivity of a copper catalyzed cyclopropanation reaction can be controlled using a chiral dithienylethene ligand additive.$^{32}$

![Scheme 1.10](image)

**Scheme 1.10** – The product distribution of a cyclopropanation reaction between ethyl diazoacetate and styrene in the presence of a copper-DTE complex is controlled using light.$^{32}$

The flexible geometry of the ring-open isomer, allows for the chiral oxazoline groups to rotate and chelate to the copper ion. The close proximity of the chiral substituents to the copper reaction site imparts a higher degree of stereoselectivity during the cyclopropanation reaction in comparison to the ring-closed isomer.

**1.3.5 Modulation of diarylethene electronic communication with light**

Another interesting feature observed with the diarylethene class of photoswitches, is that the degree of electronic communication between the substituents appended to the heteroaryl core, can be reversibly modified with light isomerization.$^{33}$ In the ring-open isomer, electronic communication is possible between substituents on the
same ring, (Scheme 1.11), and therefore R\textsubscript{1} and R\textsubscript{2} (as well as R\textsubscript{3} and R\textsubscript{4}) are connected through the thiophene π-system. The flexibility of the thiophene groups, limits the degree of electronic connection between the substituents on the opposite ring system, and for example R\textsubscript{1} and R\textsubscript{4} are said to be electronically insulated from one another.

![Scheme 1.11](image)

**Scheme 1.11** – Comparison of the electronic differences between the ring-open and ring-closed isomers. In the ring-closed isomer, conjugation is extended across the backbone of the diarylethene core, allowing for communication between the external groups R\textsubscript{1} and R\textsubscript{4}. Whereas in the ring-open isomer, the groups appended to the same π-system, R\textsubscript{1} and R\textsubscript{2} are electronically connected.

Isomerization to the ring-closed isomer changes the electronic distribution of the π-system.\textsuperscript{34} The new α-bond formed at the two positions on thiophene, creates a nearly planar orientation with extended conjugation along the backbone of the DTE. The external substituents, R\textsubscript{1} and R\textsubscript{4}, are therefore electronically connected (highlighted in bold in the figure). Additionally, as the bond hybridization of the C2 carbon changes from sp\textsuperscript{2} to sp\textsuperscript{3}, the thiophene π-system is disrupted, limiting the electronic connection between R\textsubscript{1} and R\textsubscript{2} (and between R\textsubscript{3} and R\textsubscript{4}).
The electronic differences that occur with photoswitching can be exploited to influence the behaviour of the DTE system. A few examples are given below to illustrate different methods and to control the electronic properties of the diarylethene using light.

**Controlling pyridine nucleophilicity using light:**

In the example illustrated below, the DTE architecture was used to externally control the nucleophilicity of a pyridine group using light. The thiophene external positions, are substituted with one pyridinium, and one free pyridine. See Scheme 1.12 for the structure. In the ring-open isomer, the pendent aryl groups are electronically isolated from one another. Whereas with exposure to 365 nm light, conversion to the ring-closed isomer generates a linearly $\pi$-conjugated backbone. The electronic communication pathway between the pyridinium and pyridine in the ring-closed isomer, subsequently leads to a decrease in the pyridine’s reactivity in a test alkylation reaction.
Scheme 1.12 - The reactivity of the pyridine group towards an electrophile can be controlled using light. In the ring-open isomer, the pyridine is electronically insulated from the effects of the electron withdrawing pyridinium group, accelerating alkylation rates. Whereas in the ring-closed isomer, the electronic communication between the pyridine site and the electron withdrawing pyridinium lowers pyridine's nucleophilic character and the reaction rate is slowed.\textsuperscript{35}

In the ring-open isomer, the pyridine behaved as a better nucleophile during the alkylation reaction with p-bromobenzyl bromide. The rate of formation of the bis-pyridinium addition product was approximately 3-fold higher than the ring-closed isomer.\textsuperscript{35} The substrate conversion rates could be reversibly slowed and accelerated by exposing the reaction mixture to alternating UV and visible light respectively.

**Light controlled release of a small molecule:**

In this example, presented in the scheme below, the degree of electronic communication between a p-dimethylaminophenyl group and the t-butylicarbonate group appended to the external positions of the DTE core, influences the rate of cleavage of the t-butylicarbonate group.\textsuperscript{36} In the ring-open isomer, the two groups are electronically insulated from one another and the t-butylicarbonate group is stable. However, after UV light induced conversion to the ring-closed isomer, the nitrogen lone pair of the dimethylamino substituent can donate electron density through the extended $\pi$-system. This induces spontaneous cleavage (in the dark) of the bond connecting the t-butylicarbonate to thiophene, creating the cationic quinoidal species, as well as t-butoxide and carbon dioxide. The rate of cleavage can be increased by exposure to light.
Scheme 1.13 – Light is used to increase the degree of electronic communication across the DTE backbone, which induces cleavage of the t-butylcarbonate group.\textsuperscript{36}

Photoswitchable catalysis using an N-heterocyclic carbene based dithienylethene:

In the example below, Bielawski \textit{et al}, show how the changes in electronic distribution of a photoresponsive dithienylethene catalyst can be used to alter the rate of a condensation reaction.\textsuperscript{37} In the ring-open isomer, the carbene efficiently catalyzes the amidation reaction between ethyl acetate and aminoethanol. However, in the ring-closed isomer, the electron density at the carbene site is delocalized throughout the molecule’s structure, creating a catalytically inactive species. The substrate conversion rate was approximately 100 times faster in the ring-open isomer versus the ring-closed isomer.
Scheme 1.14 – Light induced changes in electron density at the imidalozium bridgehead allow for control over the rate of amide formation. The ring-open isomer behaves as an efficient catalyst, whereas the ring-closed isomer is less reactive due to delocalization of the carbene electron density throughout the DTE core.\textsuperscript{37}

1.4 Controlling biological processes with synthetically derived molecular photoswitches

Biologically relevant molecules that are not inherently sensitive to light, can be rendered light responsive through incorporation of an appropriate chromophore into their structure.\textsuperscript{2,6} The chromophore must be able to absorb light of a specific wavelength, undergo a relevant change in structure and/or properties including geometry, polarity, and electronic structure etc. so that light energy can ultimately be converted into a useful signal to influence a biological pathway. To date, there are a number of strategies that have been developed to introduce light sensitivity into biologically relevant molecules.\textsuperscript{4,13,14,38} The general categories include:

1) Optogenetics: This approach involves introduction of genes that naturally encode for light responsive photoreceptor proteins, (i.e. microbial opsins) into cells that do not naturally produce them.

2) Photocages: This approach involves the incorporation of a photolabile protecting group to suppress biological activity, either by sterically limiting access to important functional groups, or by rendering reactive functionalities chemically inert. Activity can be selectively restored through light induced cleavage of the photolabile bond.
3) **Photoswitches:** This approach uses photoresponsive molecules, which can exist in two different isomeric states. Each state possesses a unique set of chemical and physical properties, consequently influencing their ability to interact with target biomolecules.

The work presented in this thesis focuses on using synthetically derived photoresponsive molecules. Genetically encoded photoreceptor proteins and photocaged systems will only be mentioned briefly here for comparison. Synthetic photoswitchable molecules offer a number of advantages including:

- They are small in size and relatively less structurally complex in comparison to proteins
- Synthetically accessible (typically can be prepared and isolated in large quantities)
- Tolerant to structural modifications. Functional groups can be introduced to enhance compatibility or recognition by a desired target. As well, their photophysical properties can be tuned to suit a desired application (i.e. light activation kinetics and the wavelength of light required to induce photoisomerization).
- Reversible control over activation and deactivation is possible with light.

The ability to achieve reversible control over a process using an external light source is what sets photoswitchable molecules apart from small molecule probes, i.e. photocages and non-light responsive chemical effectors. See **Figure 1.5** for a cartoon representation, comparing the activity control profiles provided with chemical means, photocages and photoswitches in a biological context."14 Chemical effectors offer little control over their activation profile once administered, represented by the green arrow at the bottom of **Figure 1.5**. In other words, these species are active at all time points. Photochemical protecting groups or chemical protecting groups, temporarily modify or block a critical feature of a biologically relevant molecule. Cleavage of the protecting can be achieved at a desired point by using light (photocages) or through metabolism (chemical protecting groups)."12 The compound remains inactive until irreversible cleavage takes place, creating the active species. Both processes are irreversible, as represented by the middle arrow in **Figure 1.5**. However, in contrast, photoswitchable molecules allow for reversible changes to be made to the structure and properties with
light irradiation (represented by the top arrow in the figure below). The feature potentially allows for multiple cycles of activation and deactivation.

Figure 1.5 - Traditional methods of control (non-light responsive chemical effectors) have a limited user defined activation profile and are in most cases active at all time points. Photoprotecting groups or photocages allow for control over the activation process, however are irreversible. Photoresponsive molecules can be reversibly toggled between active and inactive states, localized light activation allows for selective control over where a compound is active.14

Numerous examples have shown that synthetic photoresponsive groups can be designed to interact with different types of biological molecules and their function can be controlled using an external light source.6 A few select examples are reported later on in the chapter. Generally, controlling biological processes with photoswitchable molecules can be divided into the following two categories:

1) Direct – The biological molecule of interest is modified with a photoresponsive molecule, Figure 1.6 (top). This approach involves attachment of a photoresponsive group to one or more sites on the biomolecule, typically through a covalent bond. The photoswitchable group usually contains a reactive site, capable of undergoing condensation with an amino acid group, in the vicinity of an active site (or other critical area required for normal function).
2) **Indirect** – An external effector molecule is modified with a photoresponsive group [Figure 1.6](bottom). The photoresponsive molecule is incorporated into the structure of an inhibitor, activator or cofactor that can interact with the biomolecule. Changes that take place to the photoresponsive molecule indirectly influences the activity of the biomolecule through differences in their affinity for a particular site.

**direct** – photochromic group is tethered to the biomolecule

![Diagram](light controlled target structure)

- Structure in tact
- Structure distorted

**indirect** – external molecule modified with a photochromic group

![Diagram](light controlled target interaction)

- Higher affinity
- Lower affinity

**Figure 1.6** - (a) A photoresponsive group is introduced at one or more sites within the biological target, (PDB ID: 2M0Z). The reversible structural changes that occur with isomerization influence the conformation and function of the macromolecule. (b) The photoresponsive group is introduced into an external ligand. The structure and properties of the ligand are altered with isomerization and each isomer has a different affinity for the target, (PDB ID: 4CSV).
In the direct approach, the changes to the structure of photoresponsive group that take place with light absorption, are translated into changes to 1) the structure of the biomolecule by disrupting the native folded state, or 2) through physical blockage of a region of the molecule critical for function through steric interactions. As Figure 1.6 (top) illustrates, an azobenzene is tethered directly to a protein (in this case via linkage to two cysteine residues). When the azobenzene is in the cis form, the distance between the attachment sites is ideal for conservation of the protein’s alpha helical structure. Whereas when the trans isomer is created, the distance between the connection sites increases, leading to distortion of protein’s alpha helical structure.

Ideally, the light induced isomerisation reaction will reversibly distort the biomolecule in such a way, that it hinders normal function in only one of the two isomeric states. Thus, allowing the user to turn a biological process ‘on’ or ‘off’ with light illumination. This method has had an overwhelming amount of success, especially in the field of neurobiology. However, as was mentioned earlier, the attachment process can be challenging, deciding on an appropriate area to position the photoresponsive group so that structure or function can be modified in a desired way. Additionally, biomolecules are flexible and can absorb small changes in protein structure induced by photoswitching, limiting the amount of perturbation possible with light.

The ‘indirect’ approach takes advantage of the light induced changes that occur within a small molecule effector, (i.e. the geometry, dipole moment, or electronic structure) to influence the activity of the targeted biological molecule or process. Many biological macromolecules have binding sites to enable efficient interactions with effector molecules. Their binding is frequently used as a signal to trigger changes and events required for normal cellular function. The affinity of a an effector or ligand for a target system is dependent on the its overall 3-D geometry and the sum of the electrostatic interactions, hydrogen bonds, and van der Waals contacts formed. Small structural variations to an effector molecule can have a dramatic influence on the affinity for a target biomolecule. A small molecule effector, with a photoresponsive group incorporated into their structure provides a unique method to make localized changes to its structure on command and influence the binding affinity externally. Figure 1.6 (bottom) displays a cartoon example illustrating this concept. An azobenzene
incorporated into an inhibitor and light can be used to change the conformation from the \textit{trans} isomer (extended form) a geometry that favours binding to the \textit{cis} isomer (condensed form) which is too bulky to bind favourable with the active site. The work presented in this thesis focuses on the concept of using the indirect approach, where small molecules modified with photoresponsive groups are created to allow for unique changes in their structure and function to be made using light.

1.5 Selected examples of synthetically derived photoswitchable molecules for controlling biologically relevant processes

Section 1.3 described the diarylethene class of photoswitches, detailing their photochemical behaviour, structural features, and how some of their properties change with light. Specific examples of how the changes in the 3-D configuration (or geometry) and the system’s electronic distribution that take place with photoswitching have been used by researchers for different functional applications were also included. This section of the chapter includes a few select examples of how light and photoresponsive molecules are combined to influence the outcome of different biological processes, highlighting their practicality and limitations.

**Light controlled acetylcholinesterase inhibitor:**

In the example below, Trauner \textit{et. al.} describe a photoswitchable version of the drug tacrine, which inhibits the enzyme acetylcholinesterase, (AChE). This enzyme is responsible for catalyzing the hydrolysis of acetylcholine, an important reaction for synaptic transmission.\textsuperscript{42} The researchers appended the azobenzene group to the small molecule drug, tacrine, through an ethylene linker and measured its ability to inhibit AChE activity. Although, some potency was lost by appending the azobenzene to the drug molecule, the $K_i$ was measured to be 95.4 nM for the \textit{trans} isomer (compared to 6.4 nM for tacrine). The $K_i$ value for the \textit{cis} isomer could not be determined directly, due to the instability of the \textit{cis} isomer. At 1 \textmu M, the researchers demonstrated that hydrolysis could be slowed 4-5 fold by conversion of the \textit{trans} isomer.
Scheme 1.15 – Light controlled affinity of a tacrine modified azobenzene photoswitch. The incorporation of an azobenzene group into the inhibitor structure allows for reversible changes in enzyme activity to be achieved using light.42

Both isomers inhibited the enzyme, the cis isomer was found reduce activity to a larger extent than the trans isomer. The trans isomer reduced activity to 17%, compared to the control, whereas the cis isomer reduced activity to 4%, relative to the control.

Light controlled RET kinase inhibitor:

The example below features an azobenzene based photocontrolled RET (also known as REarranged during Transfection) kinase inhibitor. A photoresponsive azobenzene was attached to a pyrazolopyrimidine unit, allowing for changes in the spatial re-arrangement of the core structure.43 In the trans isomer, the linear structure binds to the active site with a higher affinity. In both cell-free and live-cell assays exhibits a higher percentage of inhibition relative to the cis isomer. An approximate 4-fold difference in activity was achieved in the cell-free assay, and a ~3-fold difference measured in the live-cell assay.
Scheme 1.16 – Light controlled activity of an azobenzene modified pyrazolopyrimidine RET kinase inhibitor.\textsuperscript{43}

A limiting feature with this system is the maximum ratio of \textit{cis} to \textit{trans} azobenzene does not exceed 87\% \textit{cis}, therefore the more active \textit{trans} isomer will always be present in at least >13\%.\textsuperscript{43} Completely turning “off” the inhibitory effects of the \textit{trans} isomer was not possible.

**Light controlled spiropyran based antimicrobial derivative:**

In this example, presented by Feringa \textit{et al}, the antimicrobial drug ciprofloxacin is appended to a spiropyran group through an amide linkage formed between the secondary amino of the piperazine ring (see Scheme 1.17 below for the structure)\textsuperscript{44}. The resulting compound exhibited higher antimicrobial activity in the merocyanine state when tested against the Gram-negative species, \textit{E. coli}. A two-fold difference in minimal inhibitory concentration (MIC) was reported in the studies. No difference was observed between the two photoisomers when tested for activity against the Gram positive organism, \textit{M. luteus}.

Scheme 1.17 – Light controlled antimicrobial activity of a spiropyran modified version of ciprofloxacin. The photoresponsive derivative allowed for reversible changes in activity against \textit{E. coli}\textsuperscript{44}.

<table>
<thead>
<tr>
<th>organism</th>
<th>MIC ((\mu)M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>spiropyran</td>
</tr>
<tr>
<td>\textit{E. coli}</td>
<td>1.25</td>
</tr>
<tr>
<td>\textit{M. luteus}</td>
<td>&gt; 2.50</td>
</tr>
</tbody>
</table>

The difference in antimicrobial activity was attributed to the polarity change that takes place with photoswitching, influencing its rate of cellular uptake.

**Light controlled Phosphoribosyl Isomerase A (PriA) inhibitor:**
In the example below, König et al, describe a series of symmetric photoresponsive phosphoribosyl isomerase A (PriA) inhibitors. The enzyme has a symmetric barrel shaped scaffold, with two binding sites on opposite sides. The inhibitor design consists of a central dithienylethene scaffold, with two pendant phosphate binding groups. The distance between the two groups and their relative orientation can be modified using light. In the ring-open form, the meta-substituted bisphosphate inhibitor displayed an IC$_{50}$ of 0.55 µM, which was eightfold more potent than the corresponding ring-closed isomer, which displayed an IC$_{50}$ of 4.4 µM. The decrease in affinity of the ring-closed isomer is attributed the shortened binding distance possible between the two pendant phosphate groups. The activity difference between the ring-open and ring-closed isomers of ortho and para-substituted derivatives was much smaller.

![Scheme 1.18](image)

<table>
<thead>
<tr>
<th>phosphate substitution</th>
<th>inhibitory activity IC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ring-open</td>
</tr>
<tr>
<td>ortho</td>
<td>8.1 ± 2.1</td>
</tr>
<tr>
<td>meta</td>
<td>0.55 ± 0.12</td>
</tr>
<tr>
<td>para</td>
<td>3.5 ± 0.3</td>
</tr>
</tbody>
</table>

**Scheme 1.18** – The activity of a bidentate Phosphoribosyl Isomerase A (PriA) inhibitor can be controlled by modifying the distance between the chelation sites. The largest change in activity is observed for the meta-substituted bisphosphate derivative, allowing for an approximate 8-fold difference in enzyme inhibition to be achieved with light.

**Light controlled ion channel activity:**

In a publication by Woolley et al, they demonstrate how red light can be used to influence the activity of an ion channel, in an azobenzene modified ionotrophic glutamate receptor. The azobenzene photoswitch contains a thiol-reactive maleimide group for
covalent attachment to an engineered cysteine residue located near the glutamate-binding site of the receptor. The terminal end of the azobenzene has a pendent glutamate substituent to mimic the effects of the natural glutamate agonist, see Scheme 1.19. When the azobenzene is in the *trans* state, the glutamate is positioned away from the binding site of the receptor, and the channel remains closed. However, with 630 nm light irradiation, the *cis* isomer is formed and the glutamate faces towards the channel. In this orientation, the glutamate group can easily access the binding site. The *cis* isomer, mimics the effects of a rapid increase in glutamate concentration, which causes the channel to open.
Scheme 1.19 – Linkage of a glutamate modified azobenzene derivative to a cysteine residue on an ionotrophic glutamate receptor, allows for light induced changes in its apparent concentration. In the trans isomer, the glutamate residue is directed away from the binding site in the channel. Conversion to the cis isomer, forces the glutamate to be positioned toward the receptor-binding site. The increase in concentration of glutamate causes the channel to open.46

1.6 Thesis outline

Two different approaches are included in this thesis to demonstrate how the light induced changes in the diarylethene scaffold can be used as a means to control the properties of biologically inspired small molecules.

The first system, featured in Chapter 2, uses the changes in the diarylethene geometry that accompany photoswitching to influence the activity of a photoresponsive protein kinase inhibitor. The inhibitor design is inspired by the bisarylmaleimide family of natural products. This scaffold was chosen because it has been shown to exhibit potent and selective protein kinase inhibition and the unique flipped binding geometry is believed to be responsible for the molecule’s potency. See cartoon illustration included in the concept figure below (top). Small structural changes to the scaffold can have a dramatic influence on how the inhibitor interacts with the enzyme target. Therefore, we hypothesized that the inherent changes that occur to the diarylethene core, with photoswitching, can be exploited to influence the molecule’s geometrical configuration and subsequently, the binding affinity for the protein kinase target.

The chapter initially outlines the rational design, development and key considerations involved in the preparation of three photoresponsive inhibitors inspired by the bisindolylmaleimide family of natural products. However, a number of challenges were encountered during the course of the project, including the poor photoswitching performance and water solubility of the inhibitor candidates.
bisarylmaleimide inhibitor:

light controlled inhibitor geometry:

mechanism 1: classic inhibition

mechanism 2: aggregate-induced inhibition
Figure 1.7 – Top: Bisarylmaleimide co-crystallized with PKC βII, the zoom in displays the flipped binding orientation in the active site (PKC βII structure, PDB ID: 2I0E). Bottom: Light controlled inhibitor design that incorporates both photoswitching and pharmacophoric features. In mechanism 1, the ring-closed isomer is locked in a planar conformation and as a result cannot adopt the preferred binding orientation in the enzyme active site. The ring-open isomer, is flexible and can re-arrange to the preferred flipped geometry, leading to a high inhibitory activity relative to the ring-closed isomer. In mechanism 2, the ring-closed isomer self-assembles into relatively large aggregates in aqueous solution. The ring-open isomer remains soluble. The inhibitory activity is higher in the ring-closed isomer believed to be due to aggregate induced inhibition, where the aggregates adsorb and sequester the enzyme, altering its catalytic ability.

Unexpectedly, photocontrol over inhibitor potency was possible through two different mechanisms. One derivative exhibited good inhibition (in vitro) that could be switched ‘on’ with brief exposure to visible light. Although, the modifications made to the photosensitive inhibitor structure to improve the photochemical performance, ultimately lead to reduced inhibitory potency (compared to the traditional bisarylmaleimide scaffold). Another derivative exhibited conformation dependent solubility in water, that is, one photoisomer (the ring-open form) was soluble and stable in aqueous media. The ring-closed isomer self-assembled into large aggregate particles, shortly after its dispersion into water. Although the inhibition mechanism is non-specific, the aggregate particles inhibited protein kinase activity. The aggregate species could be efficiently disrupted by conversion to the water soluble ring-open isomer, through brief exposure to visible light. This allowed for restoration of enzyme activity.

The second system, featured in Chapter 3, explores how the light induced changes in the diarylethene core can be used as an alternative means to influence the outcome of a chemical reaction. The photoswitch design is inspired by the naturally occurring enzyme cofactor, Pyridoxal 5’-phosphate. This diverse cofactor utilizes a change in the electronic distribution between a pyridinium and an aldehyde to catalyze a range of amino acid transformations. A simplified photosensitive version was designed and synthesized in the chapter, see Figure 1.8 below for the structure. The photosensitive PLP mimic can be transformed from a relatively inactive state, the ring-open isomer, where communication between the pyridinium and aldehyde is minimal
(and the ability to convert a substrate to product is poor) to an active state with extended electronic communication, capable of catalysis.

![Diagram of enzyme cofactor mimic](image.png)

**Figure 1.8** – The light controlled enzyme cofactor mimic inspired by Pyridoxal 5'-phosphate (right). The design incorporates two critical functional groups for catalysis; an electron withdrawing pyridinium and an aldehyde active site, onto the backbone of a photoresponsive diarylethene. The degree of electronic communication is more extensive in the active isomer, generated with UV light. The active isomer is able to convert substrate to product at a faster rate than the inactive isomer (generated with visible light) during a PLP type amino acid racemization reaction. (Image, was reprinted with permission from John Wiley & Sons, Inc.)

The work included in this thesis is meant to highlight the usefulness of developing externally controllable molecular tools and I would like to emphasize that the photocontrolled applications presented in this thesis are far from being useful in any clinical application. Rather the work presented in this thesis is meant to demonstrate how light can be used to control molecular structure of a small molecule, influencing the geometry and electronic structure and highlight its convenience as an external stimulus allowing for rapid changes to be made in structure, potentially allowing a unique method to remotely trigger events without addition of chemicals.
2 Using light induced changes in diarylethene geometry to influence protein kinase function

Some of the work presented in this chapter was recently submitted for publication in the following manuscript: Danielle Wilson, Jason W. Li and Neil R. Branda, titled: *Visible Light Triggered Activation of Protein Kinase Inhibitor*. The synthesis, physical characterization, photochemical characterization and stability analysis were designed and conducted by Danielle Wilson. Danielle Wilson and Jason W. Li conducted enzyme activity studies. The manuscript was co-written by Danielle Wilson and Neil R. Branda. Neil R. Branda designed the project concept.

2.1 Introduction

The focus of this chapter is to use the diarylethene photoswitch as a platform to introduce light responsiveness into a protein kinase inhibitor. Particularly, to investigate if predictable light induced changes in the inhibitor’s geometry can be harnessed to influence the function of a biological target of interest, in this case, protein kinase C (PKC). A light controlled protein kinase inhibitor can be created by combining the appropriate structural features that support binding to the target of interest, with the suitable functional groups that will favour light triggered photoswitching within the diarylethene unit.

The protein kinase family is large, (>500 members) and the structure of the catalytic domain is highly conserved.49 As a consequence, the design of selective inhibitors is particularly challenging and off-target toxicity often limits their usage. Recent developments in light activated enzyme inhibitors, as well as the current challenges in selective inhibition of kinases, prompted us to look into creating light controlled small molecule protein kinase inhibitors. The site specific activation of inhibitors is a relatively
unexplored area that may prove beneficial for elucidating complex pathways involved in protein kinase signal transduction.

The figure below illustrates the concept of controlling an inhibitor’s geometry with light, and consequently, altering its binding affinity for the kinase target. One photoisomer has an appropriate geometrical configuration, that is highly complementary to the active site, permitting efficient binding and inhibiting enzyme function. Whereas the second photoisomer possesses a different shape, that abolishes its affinity for the active site. In the latter process, the enzyme can function normally. Light can be used as a method to interconvert between the two isomeric states, allowing for external control over the activity of the enzyme.

**Figure 2.1** – Light induced changes in the geometry of a photoswitchable inhibitor containing a diarylethene core. The 3-D geometry of the inhibitor strongly influences the binding affinity for the target enzyme. One isomer, (yellow), generated with exposure to light of wavelength $h\nu_2$, binds to the kinase, inhibiting function. Whereas the other isomer (gray), generated with $h\nu_1$, does not bind efficiently to the target, and kinase function proceeds normally. (PKC βII, PDB ID: 2I0E).47

This chapter first includes a brief introduction to protein kinases, including an overview of their structure, function and existing challenges associated with their control. Emphasis is placed on PKC, the target of interest in this project, and how manipulation with light can provide a valuable addition to the current tools available for investigating its properties. The following sections of the chapter, focus on the photoresponsive inhibitor design process, how control over PKC function can be suitably addressed with
light induced changes in the inhibitor’s geometry, and important photophysical features to consider when preparing photoswitches that are to be compatible with biosystems. The rational design and synthesis of a series of photoswitchable inhibitors based on the bisindolylmaleimide natural product scaffold is included. Their photochemical performance will be evaluated and discussed. Finally, evaluation of inhibitory activity and photocontrol over PKC activity, using two unique mechanisms, will be demonstrated. Emphasis is placed on photochemical performance, and the common challenges and strategies associated with preparing photoswitchable bio-compatible small molecule effectors.

2.2 Protein Kinases

This section includes a general introduction to protein kinase function and the important role that protein kinases play in the regulation of cell metabolism. The target biomolecule, PKC is discussed in more detail; including how it is regulated by cofactors, which is relevant to later sections involving in vitro enzyme assays during inhibitor screening.

2.2.1 General introduction and function

Protein kinases are a family of enzymes that catalyze the transfer of a γ-phosphate from adenosine triphosphate (ATP) to a protein or a peptide substrate. In most instances, the phosphorylation site on the substrate is an alcohol group present on the side chain of either a tyrosine, serine or threonine residue. The opposite process, removal of a phosphate group, is catalyzed by protein phosphatases. These two processes are important post-translational modifications required for signal transduction in the cell.
A variety of processes rely on phosphorylation events, including apoptosis, cell cycle regulation, neurotransmission, immune responses, carbohydrate and lipid metabolism, DNA transcription and replication, and smooth muscle contraction.\textsuperscript{52} Introduction of a phosphate group to a substrate alters the molecule’s charge distribution.\textsuperscript{53} This can lead to a change in the substrate’s structural conformation or its binding affinity for other cellular components, and leads to association or dissociation events.

\subsection*{2.2.2 The Protein Kinase Family}

The protein kinase family is large and consists of at least 518 members (likely with more undiscovered protein kinases estimated).\textsuperscript{49} Protein kinases account for \(~1.7\%\) of all human genes. Kinases can be further subdivided into seven major groups:\textsuperscript{54}

1. AGC (protein kinase A, protein kinase G, and protein kinase C)
2. CAMK (calcium and calmodulin regulated kinases)
3. CK1 (casein kinase 1)
4. CMGC (cyclin-dependent kinases (CDKs), Mitogen-activated kinases (MAPKs), Glycogen synthase kinases (GSKs), CDK-like kinases (CLKs))
5. STE (Homologs of yeast Sterile 7, Sterile 11, Sterile 20 kinases)
6. TK (tyrosine kinases)
7. TKL (tyrosine kinase-like).
The classification of kinases into these different groups, is based on their sequence, structure and biological function. See below for the sub-group classification of the human kinome.

Figure 2.2 – Map of the human kinome, classified into seven major groups. The different colours represent the 7 protein kinase families. Each node represents a unique protein kinase. Names of each kinase are not included for clarity. Illustration reproduced courtesy of Cell Signaling Technology, Inc. (www.cellsignal.com).

2.2.3 The Catalytic domain and the ATP Binding Site

All protein kinases carry out the same phosphotransfer reaction, and therefore, have a similar catalytic domain. The protein kinase catalytic domain contains a binding pocket for the phosphate donor ATP, as well as a groove along the surface to accommodate the phosphoacceptor region of substrate. The catalytic subunit is made up of two domains: the C-lobe, composed of primarily α-helixes and the N-lobe, composed of mostly β-strands. The two domains are joined together by a small linkage, known as the hinge region. This section is where the binding pocket for ATP is located.
The substrate binding region is located along the surface of the kinase, just outside of the hinge, see Figure 2.3 for an example of a protein kinase catalytic domain.\cite{58}

![Figure 2.3](image)

**Figure 2.3** – (a) Protein kinase catalytic domain, showing the C lobe (pink), N lobe (green), ATP binding site with ATP bound, the hinge region (yellow) and substrate binding site (blue). (b) Zoom in of the ATP binding pocket.\cite{58} (PDB ID: 4XW5,\cite{58} from PKA, created with Pymol).

2.2.4 Protein Kinase C structure, classification and activation

PKC does not refer to a single enzyme, but rather a family of enzymes, made up of several isoforms. Currently, there are at least 11 different isoforms discovered to date, they include: $\alpha$, $\beta$I, $\beta$II, $\gamma$, $\delta$, $\epsilon$, $\eta$, $\lambda$, $\iota$, $\zeta$, and $\theta$.\cite{59} The isoforms differ in their regulatory domain sequence and the cofactors that they require for activation into a catalytically competent enzyme. They are divided into three different groups: classic, novel and atypical. PKC-$\alpha$, $\beta$I, $\beta$II and $\gamma$ are referred to as the classic PKC isoforms and they all require diacylglycerol (DAG) or alternatively, a Phorbol ester, as well as calcium, and phosphatidylserine for their activation.\cite{59} The novel PKC isoforms include PKC-$\delta$, $\epsilon$, $\eta$, and $\theta$ and require DAG (or a Phorbol ester), and phosphatidylserine for activation. However, the novel isoforms and function independently of calcium. The last group of PKCs are the atypical isoforms, which include PKC $\lambda$, $\iota$, $\zeta$. This particular group does not require either DAG (or Phorbol esters) nor calcium for their activation, and only respond
to phosphatidylserine. See Figure 2.4 for an illustration of the different domains that make up the classic, novel and atypical isoforms.

**Figure 2.4** – Classification of protein kinase C isoforms based on their cofactor requirements. There are currently 11 known PKC isotypes, which fall into three classes: classical (α, βI, βII, γ), novel (δ, ε, η, and θ) and atypical (ζ, ι, λ). Each class has a characteristic domain structure based on the presence and/or sequence of the C1 domains, C2 domains, and pseudosubstrate site (PS) and catalytic domains. The C1B domain is from PKC α (PDB ID: 2ELI), C2 domain from PKC α (PDB ID: 1DSY) and C3 and C4 from PKC βII (PDB ID: 2I0E).

All PKCs share a similar catalytic domain, illustrated as C4 and C3 in Figure 2.4. The regulatory domain, however, differs amongst the three classes. In the classical PKCs, the regulatory C1A and B domains are located in the N-terminal region of the protein. The C1 region, represents the Phorbol ester/diacylglycerol binding sites, and the C2 region, represents the calcium binding sites. The novel PKCs contain a C1 and C2 domain, however, their order is reversed in comparison to the classical isoforms, with the C2 domain closest to the N terminus, followed by the pseudosubstrate site, and the C1 domains respectively. Additionally, the C2 domain differs in the novel PKC isotypes, in that it does not coordinate to calcium ions, and its activation has been proven to be independent of calcium. Atypical PKCs differ from classical and novel isotypes, in that they function independently of calcium, diacylglycerol or Phorbol esters. They do not
have a C2 region for binding calcium. A C1-like region is present, although it is structurally different from the C1 found in the classical and novel PKCs.

All PKC isotypes contain an autoinhibitory sequence in the N-terminal region of the regulatory domain, labelled as PS in Figure 2.4. The pseudosubstrate resembles a typical PKC substrate, however does not contain a phosphate-accepting residue. Like the name suggests, the autoinhibitory sequence binds to the kinase in the substrate binding region, and acts as an inhibitor preventing a real substrate from entering and being phosphorylated. It’s purpose is to keep PKC in its condensed (inactive) form, where it is less prone to cleavage by proteases. The autoinhibitory pseudosubstrate remains bound to the enzyme catalytic domain, maintaining a catalytically inactive enzyme, until the appropriate concentrations of cofactor signals (calcium, DAG or phosphatidylinositol) are released in the cell. These cofactors cause translocation of the inactive enzyme from the cytosol to the cell membrane, which upon binding cause the pseudosubstrate to dissociate from the substrate binding pocket. Release of the pseudosubstrate is accompanied by a large structural rearrangement to the open, catalytically active enzyme.

2.3 Photoresponsive inhibitor design

This section describes the properties of the bisarylmaleimide family of protein kinase inhibitors, detailing the structure activity relationships available for existing bisarylmaleimide derivatives. Next, the rationale behind our photoresponsive hybrid design is presented.

2.3.1 Inhibitor structural inspiration

2.3.1.1 Bisarylmaleimides as protein kinase inhibitors

We utilized an existing inhibitor scaffold, the bisarylmaleimides, with well defined structure activity relationships (SAR) and interesting structural features that can be exploited with light. The 2,3-bis(indol-3-yl)maleimide family of alkaloids, also commonly referred to as bisindolylmaleimides, (or BIMs) was selected as a starting point for the
design of the light controlled inhibitor system. Like the name suggests, the basic core structure contains a central maleimide group substituted with two indole moieties, which are connected to a central maleimide, at the 3,3'-positions. The BIM scaffold and other structurally related derivatives have been isolated from several different species of prokaryotes and eukaryotes. Many have been shown to have interesting and diverse biological activity profiles, for example, as antimicrobials, anti-inflammatory agents, and nanomolar inhibitors of some serine/threonine protein kinases. The Arcyriarubin family of compounds, isolated from the Myxomycetes species of slime moulds, are a well known example of bisindolylmaleimide core found in nature (a few examples are shown in Figure 2.5). These and other related species have been proven to be fairly potent inhibitors of PKC, and show moderate antimicrobial activity. Diverse substitution patterns on the indole ring and indole nitrogen have been identified in nature or have been synthesized in attempts to enhance inhibitory potency and selectivity.

A number of BIMs were later discovered to have increased selectivity profiles for some protein kinases targets over others, for example displaying low nanomolar binding constants for PKC, see later section for some examples. The selective nature of the BIM scaffold for certain protein kinase targets is interesting from a medicinal chemistry standpoint. The highly conserved catalytic domain among the protein kinase family, makes the selective targeting of one particular protein kinase, over another, challenging. As a result, the high potency and selectivity discovered amongst the bisindolylmaleimides towards PKCs, has prompted interest from both academia and industry to further explore the BIM structure-activity relationships. They were later discovered to be ATP competitive and presumably exert their inhibitory activity by
preventing phosphorylation of protein substrate.\textsuperscript{68b} To date, numerous optimized derivatives have been developed in an effort to create a suitable drug candidate. Some BIM candidates have entered clinical trials for treatment of various PKC aberrant diseases ranging from carcinoma to diabetic retinopathy.\textsuperscript{71}

The reason behind selection of the BIM scaffold for our photoswitchable inhibitor design is two-fold, and originates from their unique structure and binding orientation. They contain a 1,3,5-hexatriene system, where the indole groups are connected at the 3,3’-position to the central maleimide double bond, creating a system of alternating double and single bonds. See Figure 2.6 below, highlighting the 1,3,5-hexatriene system of BIM and several other structurally related photoresponsive molecules. This structural resemblance to the ring-open isomers of diarylethenes and fulgides, suggests the possibility for photochromism (though does not guarantee efficient photoswitching performance), and could provide a means to externally control the structure, geometry, and ideally the inhibitory functionality of the BIM molecule with light energy.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2_6.png}
\caption{The bisindolylmaleimide family contain a 1,3,5-hexatriene scaffold, that resemble the well known P-type photoswitches, such as diarylethenes and fulgides.}
\end{figure}

Similarly to diarylethenes or fulgides, the BIM scaffold can absorb light and undergo a $4n + 2\pi$ electrocyclization reaction to produce a “ring-closed” isomer. We hypothesized that the same electrocyclization process could be used in the bisindolylmaleimide series, and the structural or 3-D geometrical changes that occur with
photoswitching can be exploited to create a light controlled inhibitor with PKC affinity that is dependent on the photoisomerization state.

In addition to their photoresponsive ability, and what intrigued us the most, was the unique and highly conserved ‘flipped’ binding conformation that the BIM molecule adopts in the active site, believed to be critical for its potency and selectivity for PKC.\textsuperscript{47,72} PKC has been extensively studied, and although a complete understanding of this enzyme is absent, there is substantial information available on both its behaviour and structure. A number of high resolution 3-D structures of kinases bound to BIM derivatives have been solved to date and yield a fairly consistent binding pattern. BIMs were found to bind exclusively to the ATP pocket, and in nearly all cases adopt a very similar orientation in this binding pocket. See Figure 2.7 below, for examples of several BIM derivatives co-crystallized with a protein kinase.\textsuperscript{72-75}

![Conserved 'flipped' structure observed in multiple BIM-kinase co-crystal structures](image)

**Figure 2.7** – Several overlapped 3-D structures of BIM structures co-crystallized with PKC in the ATP binding site, highlighting the conserved ‘flipped’ conformation. Structures compiled (PDB IDs: (a) 1WXS,\textsuperscript{73} (b) 2V7O,\textsuperscript{74} (c) 1UU8,\textsuperscript{72} (d) 4OTH\textsuperscript{75}).

The flexible and canted orientation of the two indole groups, relative to the maleimide (BIM inhibitor shown in gray sticks in Figure 2.7 a-d), is conserved in all of the structures displayed. The maleimide makes several hydrogen bonds (typically 3, but at least 2) with the amino acids in the hinge region of the kinase, the site where the adenine ring of ATP binds, Figure 2.8a. One indole group adopts a flipped conformation,
with a dihedral angle of ranging from -130 to -140°. The second indole occupies part of
the triphosphate group binding site, making several hydrophobic interactions with the N-
lobe and the glycine rich motif. The aminoalkyl chain, attached to the indole group,
extends outside of the binding site, and makes several electrostatic interactions in the
ribose binding site.47

Figure 2.8 – Example of a BIM derivative co-crystallized with PKC in the ATP active site.
(a) Top view illustrating the hydrogen bonds (gray dotted lines) formed
between the surrounding amino acid residues of the kinase hinge region
in the ATP binding pocket. (b) Zoom in of a side view of a BIM derivative
demonstrating the twisted indole conformation that is typically observed in
most structures resolved. The dihedral angle ~-140°. (Structure used in
the illustration from PDB ID: 2I0E).47

Interestingly, similar structures with restricted flexibility, for example the
indolocarbazole, Arcyriaflavin A (see Figure 2.9, on the next page for its structure and a
comparison to Arcyriarubin A), were shown to have a limited inhibitory effect, with IC₅₀
values ~50 µM.76 Though each of their structures contain two indole groups and a
central maleimide, the carbazole Arcyriaflavin differs from Arcyriarubin in that the two
indole groups are bridged in the at the 2,2'-position. This limits rotation to the flipped
structure, and creates a planar cyclic species. This finding helps to support our concept
that the flexible BIM geometry is important for PKC activity since the conformationally
restricted carbazole structure, demonstrates poor inhibition.
Figure 2.9 – Comparison of the structures and inhibitory activity of bisindolylmaleimide Arcyriarubin A (left) and carbazole Arcyriaflavin A (right). The two compounds have similar 2-D structures, however, the 3-D structures reveal their difference in geometry. Arcyriarubin A can adopt a twisted or flipped structure, and shows a stronger inhibitory effect on PKC, with a measured IC$_{50}$ of 0.1 µM. In contrast, Arcyriaflavin A is planar and has a measured IC$_{50}$ of ~50 µM. An approximate 500-fold difference in inhibitory activity is observed for Arcyriarubin A and Arcyriaflavin A.$^{76}$ If a similar difference in activity could be achieved with a photoresponsive version, this would be a substantial achievement in the field of photocontrolled inhibitors, which currently, there are very few examples that exist where the difference in activity is greater than 20-fold.$^{14}$ This would enhance the practical use of photoresponsive inhibitors in real applications, where full ‘on’ and ‘off’ activation or deactivation of enzyme function is desired.

2.3.1.2 Light controlled geometry and target affinity

The combination of the unique “active” binding structure and the structural resemblance to the diarylethene family, prompted us to investigate whether or not it was possible to use light energy to change the geometry in situ, and thus inhibitory activity of PKC. Scheme 2.2 below displays the predicted changes in geometry of the BIM core upon illumination with appropriate wavelengths of light. Assuming that the BIM molecule behaves in a similar manner to the diarylethene family of photoswitches, the geometry could toggled between one isomer capable of mimicking the flipped active binding geometry, to one with a conformationally restricted core, unable to adopt the desired conformation.
Scheme 2.2 – Concept of photoswitching the BIM scaffold geometry from a flexible structure, capable of adopting an active binding conformation 2.1-o to the rigid inactive isomer 2.1-c. Due to the flexibility of the ring-open isomer 2.1-o, the two pendant indole groups are free to rotate, and likely exist as an equilibrium mixture of several possible conformers. Two possible isomers are included in the scheme for illustration purposes. One isomer (far left) is the active inhibitor geometry, with one indole ‘flipped’ labeled 2.1-o \textit{flipped}. The second isomer (center) is the antiparallel conformer, labeled 2.1-o \textit{antiparallel}, from which photoisomerization can occur from.

Compound 2.1-o can adopt a range of conformations, and two of the possible structures are shown in Scheme 2.2 above; the ‘flipped’ binding conformation 2.1-o \textit{flipped}, presumably the active inhibitor and the 2.1-o \textit{antiparallel}, where the indole groups are oriented antiparallel to one another, so that photoisomerization can take place. As outlined in Chapter 1, the conformation of the aryl groups are important for photoisomerization and ring-closure can only take place from the antiparallel species.\textsuperscript{20,27} If the double bonds of the ring-open isomer are not close to one another for sufficient orbital overlap to take place, then photoisomerization to the ring-closed isomer is not possible. It is important however to emphasize that these two conformers shown for 2.1-o are not the only ones possible. Compounds 2.1-o can presumably adopt a range of conformations, and these structures are expected to exist in equilibrium.\textsuperscript{77} Calculations may be useful to predict the preferred conformational state(s) and the relative energies required to interconvert between the different isomers, however this was not the focus of the work.

Our design allows for light triggered removal of the C-C carbon bond bridge, that would otherwise prevent rotational motion of the two indole groups. It should be
emphasized however, that we can’t assume that the structure will spontaneously rearrange to the desired binding geometry once the inactive ring-closed isomer is exposed to light.

The project concept relies on the fact that the active site of most enzymes are highly specific for their substrates. The protein tertiary structure, allows for amino acids to be held in the active site in a precise arrangement in order to create a unique pocket with high electronic and geometrical complementarity with its native substrate so that binding is favoured. Frequently, binding pockets have complex surfaces which contain a number of cavities and clefts. This is true for the ATP site of protein kinases, and ATP competitive inhibitors should make a combination of favourable electrostatic, hydrogen bonds, van der Waals interactions with the pocket, in order to effectively outcompete ATP and abolish function. Changes to the 3-D structure can interfere with the number and strength of these interactions. Based on this theory we anticipate that our design will allow a significant enough change in structure with light induced isomerization, that this will influence the binding affinities of the two isomers.

2.3.1.3 Inhibitor geometry considerations

The previous section, introduced the bisarylmaleimide structure and emphasized how its flexible, slightly canted configuration is believed to be critical for the observed potency and selectivity. Based on the reported activity differences between BIMs and the more conformationally restricted carbazole scaffold, this seems to be true. However, caution must be exercised when making this prediction, since the structure-activity relationships of indolylmaleimide carbazoles and related derivatives are complex. A number of bacterial and marine metabolites, possess the indole[2,3-a]carbazole alkaloid ring system (See Figure 2.10 for a few examples). The carbazoles shown below display a range of activity, some potent, protein kinase inhibitors. For example, the related lactam carbazole, staurosporine.
Figure 2.10 - Natural product metabolites sharing the indole[2,3-a]carbazole alkaloid ring system.\textsuperscript{76,80-85}

Staurosporine, in particular, has an interesting and extensive biological profile including antifungal, hypotensive and platelet aggregation activities. Staurosporine was first isolated from Streptomyces staurosporeus (AM-2282) in 1977 by Omura et al.\textsuperscript{80} However, it inhibits a wide range of protein kinases, exhibiting little selectivity. The poor selectivity and high degree of off target activity limits its use in both therapeutics and as a tool to study enzyme function.

Interestingly, removal of the sugar group, to give the aglycone K252-c, leads to a dramatic reduction in the inhibitory activity towards PKC. A nearly 1000-fold increase in IC\textsubscript{50} is reported for K252-c compared to staurosporine.\textsuperscript{76} A summary of their activities is provided below, in Table 2.1. A range of lactam and maleimide carbazole species have been isolated or synthesized, and screened for inhibitory activity against PKC. In carbazole containing inhibitors, the combination of the sugar residue and the lactam bridgehead, appear to be critical both for \textit{in vitro} and \textit{in vivo} for PKC inhibition.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|}
\hline
Compound name & PKC activity (IC\textsubscript{50}) \\
\hline
staurosporine & \\
rebeccamycin & \\
UCN-01 & \\
K252-a & \\
tijpanazole J & \\
7-hydroxy-K252-c & \\
K252-c & \\
1,11-dichloro-arcyriaflavin A & \\
BE-13793C & \\
\hline
\end{tabular}
\caption{Summary of carbazole activity against PKC}
\end{table}
<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>7-hydroxy-K252-c</td>
<td>22.1 µM</td>
</tr>
<tr>
<td>K252-c</td>
<td>2.45 µM</td>
</tr>
<tr>
<td>Arcyriaflavin A</td>
<td>44.7 µM</td>
</tr>
<tr>
<td>1,11-Dichloroarcyriaflavin A</td>
<td>&gt;100 µM</td>
</tr>
<tr>
<td>Staurosporine</td>
<td>3 nM</td>
</tr>
<tr>
<td>UCN-01</td>
<td>29 nM</td>
</tr>
<tr>
<td>Rebeccamycin</td>
<td>&gt;100 µM</td>
</tr>
<tr>
<td>K252-a</td>
<td>32.9 nM</td>
</tr>
</tbody>
</table>

Note: Values are from a study conducted by Prudhomme et al.71c with the exception of UCN-01.86

Although a number of structural and functional distinctions are obvious with the BIM and the carbazole staurosporine, the basic core structure is not bridged at the indole-2-positions, creating a more flexible core with freely rotatable indole groups. From the table, the non-glycosylated carbazole structures, typically show weak inhibition towards PKC and related serine/threonine kinases. The ring-closed isomer, is arguably similar in some aspects, to the carbazole non-glycosylated indolocarbazole Arcyriaflavin structure.

2.3.1.4 Structure-Activity relationships in BIM protein kinase inhibitors

A number of research groups in academia and industry have diversified the bisindolylmaleimide core scaffold to improve activity, see Figure 2.11 for a few examples. Generally, the optimized design framework conserves the BIM core, and one or more the indole groups are substituted with a flexible amino alkyl chain, or the two indole groups are connected by a long flexible linker with an amino group H-bond donor (as in Ruboxistaurin, Figure 2.11).71 The amino functionality interacts electrostatically with a carboxylate group of the enzyme, typically the side chain of an aspartate or glutamate residue.68b
Figure 2.11 – Structures of bisindolylmaleimide based PKC inhibitors. (References for structures: 2-methyl-BIM,\textsuperscript{47} BIM IX and BIM X,\textsuperscript{68(c)} GF 109203X and BIM II,\textsuperscript{69} Ruboxistaurin,\textsuperscript{87} and Enzastaurin.\textsuperscript{88}

The compound GF 109203X, has been proven to selectively inhibit specific isoforms of PKC IC\textsubscript{50} in the low nanomolar range. A summary of the inhibitor SAR for GF 109203X and other BIM derivatives is given in Table 2.2.

Table 2.2 – Structure activity relationship for bisindolylmaleimides

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Activity PKC isoform (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α</td>
</tr>
<tr>
<td>GF 109203X</td>
<td>3.5</td>
</tr>
<tr>
<td>2-methyl-BIM</td>
<td>292</td>
</tr>
<tr>
<td>BIM IX</td>
<td>5</td>
</tr>
<tr>
<td>Enzastaurin</td>
<td>400</td>
</tr>
<tr>
<td>Ruboxistaurin</td>
<td>360</td>
</tr>
</tbody>
</table>
2.3.2 Photoswitchable small molecule inhibitors

The previous section outlined some of the features that are important for inhibitor design, discussing the structure-activity considerations and specific functional groups or features that the scaffold should ideally possess for maintaining active site binding interactions. However, this only satisfies half of the overall goal of the project and there are another set of important considerations when designing the photoswitchable enzyme inhibitor – the photoswitching performance. In addition to the structural features incorporated into the inhibitor, for favourable interactions with the target enzyme, the light triggered isomerization process (photochemical performance) must also be considered. The two separate design concepts can be summarized for photoswitchable inhibitors as follows:

1. The inhibitor must include key structural elements in the inhibitor design to maintain favourable interactions between the binding site on the kinase. The binding affinity should be in a range that is comparable to existing inhibitors.

2. The structure of the inhibitor must also incorporate elements to make photoswitching possible. The wavelength and irradiation conditions should also be compatible with biological systems.

Although the BIM scaffold contains the 1,3,5-hexatriene unit, also observed in the photoresponsive diarylenes, and in theory, photoisomerization is feasible. This feature alone, does not guarantee that rapid and reversible modifications of the inhibitor’s structure and potency, can be achieved using light. There is little information available regarding the photochemical performance of bisarylmaleimides, especially in a biological context. Changes to the diarylethene electronic structure can hinder or prevent photoswitching altogether. Due to this, the pharmacophoric features must be incorporated into the photoswitchable molecule in such a way, that desirable photoswitching behaviour can still be achieved. This section will outline how the features necessary for inhibitory performance were incorporated into the diarylethene scaffold.
In drug design, or inhibition of enzymes in vivo, other factors such as favourable uptake and distribution must also be considered. Additionally, inhibitors that are drug candidates should be selective and specific to the particular target of interest. This is in order to avoid potential off target side effects associated with undesired toxicity. These are not within the range of scope of this thesis chapter and will not be discussed here.

2.3.2.1 Key features for a photoresponsive inhibitor

In addition to photoisomerization, the inhibitor must include structural elements that allow for absorption of light and subsequent isomerization to a new species that has an altered structure, geometry, or electronic properties so that its ability to interact with the desired target can be “switched” efficiently using an external light source. A photoresponsive inhibitor system will ideally possess the following characteristics:

1. The inhibitor must show high potency that is at least somewhat comparable with the parent clinically useful inhibitor that inspired the design.

2. Differences in activity between the inactive and active isomers should be large enough to allow for ‘on’ and ‘off’ switching of their activity.

3. Photoisomerization should take place efficiently and with low energy light.

2.3.2.2 Creating a Hybrid BIM-DAE photoresponsive inhibitor

In order for the photoresponsive inhibitor design to have both the satisfactory photochemical and pharmacological performance, the key structural features that will support both of these goals must be identified. Unfortunately, the unmodified BIM scaffold is not an efficient photoswitch. For a number of reasons, the bisindole substitution pattern severely hinders photochemical performance. As mentioned in section 1.2 of the previous chapter, the photoswitching performance of the diarylethenes are dependent on the aryl groups substituted in the hexatriene core. Though indole is capable of undergoing light induced cyclization, to give the ring-closed isomer as observed with the traditional excellently behaved dithienylethenes, the higher aromatic stabilization energy associated with the indole group, limits the yield of the photoisomerization reaction and the stability of the photogenerated ring-closed isomer.
While BIM derivatives are excellent inhibitors, modifications will have to be made to its existing structure, in order to demonstrate light controlled inhibitory activity. A few key guiding principles can aid in narrowing the options. Firstly, identify which groups are critical to conserve suitable enzyme active site recognition and binding (or replace them with suitable residues). Second, prioritize which groups are necessary for binding and which may tolerate substitution, replacement, or modification in some way. The concept of creating photoswitchable BIMs is unexplored, and therefore, first generation structures should also be relatively synthetically accessible.

2.3.2.2.1 BIM photochemical performance

To illustrate what is meant by “poor” photochemical performance of the bisindolylmaleimide group, a UV-visible absorption spectrum below of bisindolylmaleimide derivative 2.2-o, before and after exposure to light is shown in Figure 2.12 (a). A traditional perfluorinated dithienylethene photoswitch, 2.3-o that exhibits excellent photochemical performance, is included for comparison. The details of 2.2-o are discussed later on, in the results section of the chapter. The reason for including the absorption spectrum in this section is to give the reader a sense of why modification is necessary.
Figure 2.12 – (a) Absorption spectrum of the BIM derivative 2.2-o before (gray solid line) and after (orange dotted line) irradiation to the photostationary state. The small changes to the absorption spectrum with light illumination, indicates only trace amounts of 2.2-c are produced. The inset, is a zoom in of the very small absorption band from 2.2-c that appears between 550-650 nm with 450 nm light irradiation (for a total of 7 minutes). (b) Absorption spectrum of a dithienylethene derivative, 2.3-o before (gray solid line) and after (blue dotted line) irradiation to the photostationary. Light exposure produces a dramatic change in absorption spectrum as 2.3-c is produced in excellent yield.

When a solution of 2.3-o, a dithienylethene is exposed to 312 nm light, it rapidly undergoes cyclization to yield the ring-closed isomer 2.3-c. The isomerization process is accompanied by a dramatic change in the absorption spectrum, a decrease in the intensity of the peak at 305 nm is observed, and subsequently, two new bands appear at 390 nm and 610 nm. The intensity of the new absorption bands, suggest that the photoreaction proceeds to a significant extent (photoconversion yield in this case is ~92%). However, the BIM derivative 2.2-o, exhibits miniscule changes in its absorption spectrum with light irradiation. The changes are only visible in the zoom in inset, of the 550-650 nm region. The small changes suggest that the extent of ring-closure is very small, <5%. Such a small change in isomeric ratio after light exposure, is impractical for use as a photoresponsive inhibitor. A small decrease in concentration of one isomer (in this case the active enzyme inhibitor) will not likely have any measureable effect on enzyme activity.

2.3.2.2.2 Structural design

Considering the structure activity relationship data available in literature, the free N-H maleimide is important for making several contacts with the hinge region of the ATP binding site. Typically modification of the maleimide, even seemingly small changes, for instance reduction of a C=O group of the maleimide, to a hydroxyl group, to yield the hydroxylactam is met with a substantial decrease in inhibitory activity. Substitution of the imide N-H for a methyl group, or replacement of the nitrogen with an oxygen (anhydride), results in complete abolishment of activity, see Figure 2.13 below for examples.
In terms of photochemical performance, substitution of the maleimide N-H group with bulkier phenyl groups is a proven option for increasing the photostationary state. However, substitution at the imide N-H group would prevent formation of important H-bond contacts in the enzyme active site. Therefore, the maleimide was conserved. One section of this chapter, describes a lactam system, where one of the C=O double bonds of the imide is reduced.

Also, in most instances, at least one indole group appears to be necessary for activity. To our knowledge, there are not any bisaryl maleimide, protein kinase C inhibitors reported where both indole groups were removed. Based on this, and the above table summarizing the effect of maleimide modification, the indole and maleimide groups were considered necessary for activity. Therefore, they were conserved in our design to increase the chances that the inhibitor would mimic the binding interactions formed within the active site. Substitution of one indole for another similarly sized heterocycle has been demonstrated to reduce activity, however this modification is more tolerable than manipulation of the maleimide group. As some of the indole-aryl substitutions still maintain moderate inhibitory activity. For examples, see Table 2.3, summarizing the effect of replacement of one of the indole groups with another aryl group.
Table 2.3 – Structure activity relationship for aryl modifications to BIMs

<table>
<thead>
<tr>
<th>Ar</th>
<th>IC$_{50}$ (µM)</th>
<th>Ar</th>
<th>IC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-indolyl</td>
<td>0.22 ± 0.08</td>
<td>2-napthyl</td>
<td>2.8 ± 1.2</td>
</tr>
<tr>
<td>1-methyl-3-indolyl</td>
<td>0.3 ± 0.06</td>
<td>3-benzo[b]thienyl</td>
<td>0.90 ± 0.27</td>
</tr>
<tr>
<td>1-benzyl-3-indolyl</td>
<td>1.2 ± 0.5</td>
<td>3-benzo[b]furanyl</td>
<td>2.6 ± 0.7</td>
</tr>
<tr>
<td>1-propyl-3-indolyl</td>
<td>0.48 ± 0.08</td>
<td>phenyl</td>
<td>7.7 ± 0.1</td>
</tr>
<tr>
<td>1-phenyl-3-indolyl</td>
<td>0.36 ± 0.06</td>
<td>3-pyrrolyl</td>
<td>4.0 ± 2.0</td>
</tr>
<tr>
<td>1-methyl-2-indolyl</td>
<td>&gt;100</td>
<td>3-thienyl</td>
<td>13.8 ± 3.9</td>
</tr>
<tr>
<td>1-indolyl</td>
<td>3.0 ± 0.6</td>
<td>2-thienyl</td>
<td>5.2 ± 2.2</td>
</tr>
<tr>
<td>1-napthyl</td>
<td>0.81 ± 0.21</td>
<td>3-(7-aza-1-methylindolyl)</td>
<td>2.9 ± 0.6</td>
</tr>
</tbody>
</table>

Note: Values from studies conducted by Davis et al.$^{68a}$.

Replacement of one indole groups for a naphthalene, connected at either the 1 or 2 position, leads to a decrease in potency in both cases (relative to Ar = 3-indolyl in Table 2.3). However, the magnitude of the decrease, revealed that the site of connectivity is critical for activity. The 2-napthyl group led to an approximate 13-fold decrease in activity. The IC$_{50}$ was measured to be 2.8 µM compared to the traditional 3-indolyl substituent which was 0.22 µM. Whereas, with the 1-napthyl derivative, this substitution pattern, led to a ∼4-fold decrease in activity. Additionally, the 1-methyl-2-indolyl derivative led to loss of activity, with IC$_{50}$ >100 µM.$^{68a}$ Larger deviations from the size and shape of the 3-indoyl derivatives, leads to a larger decrease in activity.
Attachment of the aryl group in the 2-position results in a significant decrease in activity. The replacement of indole with other aryl groups also led to a decrease in activity. The benzothiophene substitution led to an approximate 4-fold increase in activity with an IC$_{50}$ of 0.9 µM.\textsuperscript{68a} Whereas benzofuran, pyrrole, phenyl and thiophene all lead to IC$_{50}$ values in the low micromolar range, See Table 2.3 for comparison. The bisindole containing derivatives clearly give the highest potency, and as a result, the majority of SAR literature focuses on detailing bisindole related substitutions. The indole makes a number of important hydrophobic and van der Waals interactions with the hydrophobic outer edge of the pocket, as well as in the phosphate binding region. Even substitution at the 1-position of indole with relatively bulky groups appears to be tolerated, resulting in a small decrease in potency, for example 1-phenyl-3-indolyl led to a 1.5 fold decrease activity compared to the N-H maleimide.

In order to enhance the photochemical performance, one of the two indole groups was replaced with a more suitable aryl group. Though several alternative starting structures were considered, the thiophene and benzothiophene groups were ultimately chosen because they typically exhibit good photoswitching ability, they have a low aromatic stabilization energy in comparison to indole, and to other heterocycles such as pyrrole, furan, benzofuran etc.\textsuperscript{20} A summary of the hybrid photoresponsive inhibitor structure, featured in this chapter is given below. The conserved pharmacophoric groups highlighted in hollow orange boxes, and the modifications made to vary the photoswitching behaviour, are highlighted in the pink shaded areas.
Photoswitchable inhibitor design indolylmaleimide:

![Diagram of indolylmaleimide](image)

**Figure 2.15** – General photoresponsive inhibitor design. Hollow orange boxes represent pharmacophoric elements necessary for binding and the pink shaded areas represent features added to modify photoswitching behaviour.

Another modification that was made to the photoresponsive inhibitor structure was addition of the methyl groups on the 2 position of the pendant heterocycles. This is to prevent oxidation to the carbazole structure after ring-closure, and also to enhance the reversibility of the system.\(^\text{20}\) However, there was an additional concern that the presence of the methyl groups on each of the aryl moieties (2-position), may be sterically unacceptable, and reduce binding affinity. In the majority of BIM inhibitors reported in the literature, the 2-position on the indole group remains unsubstituted.\(^\text{68-72}\) There are no obvious advantages to having the 2-position substituted on indole, and the lack of it being tested in literature is not surprising. However, in the case of photoswitchable inhibitors, including the methyl group is critical for a number of aspects of the photochemical performance, photoisomerization yield, stability of the ring-closed isomer, and potential side product formation.\(^\text{20}\)

In a publication by Grant et al, the 2,2’-dimethyl substituted BIM (compound 2-methyl-BIM) revealed a decrease in inhibitory activity compared to the non-methylated derivative GF 109203X,\(^\text{47}\) refer to Table 2.2 in previous section for comparison of inhibitory constants. However, for the PKC \(\beta\)I and \(\beta\)II isoforms, a low nanomolar inhibitory constant was measured. Therefore, this activity comparison study suggests
that the methyl groups are tolerated by the active site in some isoforms of PKC. The remaining classic and novel isoforms, do not incorporate the additional bulk from the methyl groups as well. The atypical isoforms, both the methyl substituted and unsubstituted appear to exhibit poor inhibitory performance. The presence of the 2 methyl groups at the 2-position of indole were tolerated by some of the PKC isotypes, and were therefore, included in most of the photoswitchable inhibitor designs featured.

**Alternative inhibitor design – indolyl-lactam:**

![Diagram of alternative inhibitor design](image)

**Figure 2.16** – Structure of an alternative photoresponsive inhibitor design modifying the maleimide to a lactam to enhance photoswitching.

During the course of the project, a second alternative structure was considered for the photoresponsive inhibitor design. Instead of modifying one of the aryl groups, both indoles could be conserved, maintaining the same hydrophobic interactions as the native BIM scaffold. Although, in order to enhance the photoswitching performance, the maleimide was reduced to a lactam or a hydroxylactam. With the removal of one of the hydrogen bond donor C=O groups, just 2 H-bond contacts are possible, (instead of 3 possible with the maleimide bridge system). The activity may be reduced, however, the lactam design could provide an alternative method for our photoresponsive inhibitor design.
2.4 Type 1 derivatives – Heteroaryl analogues of bisindolylmaleimides

As was outlined earlier, the photochemical behaviour of diarelenenes, and in particular, bis-aryl maleimides is highly dependent on the electronic nature of aryl groups flanking the central alkene. Selection of an appropriate aryl moiety for the photoresponsive inhibitor design is crucial, for both photochemical performance and maintenance of inhibitory activity. Though in theory, there are many aryl groups to choose from and various substitution patterns possible for our design, for example thiophene, furan, thiazole, pyrrole, oxazole, imidazole, pyrazole, pyridine, benzo furan, benzothiophene, indole, and benzosilole have all been successfully used in various photoswitch applications. Thiophene groups present one of the most frequently encountered choice of aryl appendage in the diarelene class of photoswitches due to their exceptional photochemical performance. Their low aromatic stabilization energy that allows for efficient photoinduced ring-closure and thermal stability of the ring-closed isomer. We reasoned that to generate a photoswitchable analogue with improved photochemical behaviour, one of the indole groups of the BIM core could be replaced with a thiophene group instead. In this first series of inhibitors, we sought to investigate how a typically “well-behaved” aryl substituent choice, thiophene, would perform when the when coupled to the indolylmaleimide.

2.4.1 Design of photoresponsive thienyl indolylmaleimides

To date, there have been several reports described in the literature, detailing the photochemical behaviour of thiophene and phenyl thiophene substituted anhydrides and maleimides. However, reports on indole containing maleimides or anhydrides, and their photochemical behaviour, are far more scarce. Our analogue would serve as a starting point to investigate the photochemical behaviour; mainly the PSS, wavelength of light required for photoisomerization and stability in aqueous media. Our analogue would also allow for us to investigate if the thiophene structural modification is acceptable in terms of retaining inhibitory activity.
The inhibitor design consists of components that are either appended to the photoresponsive inhibitor to enhance enzyme binding affinity, or to improve the photoisomerization behaviour. The structural design for series 1 has four general components to consider:

**Enzyme binding** – required for interactions with protein kinase:

1. *Unsubstituted maleimide*. The maleimide N-H and carbonyl groups serve as H-bonding contacts in the PKC hinge region.

2. *One of the two indole groups*. Indole makes several hydrophobic interactions with the residues in the active site.

**Photoswitching** – required for improving photoisomerization yield:

3. *Thiophene (phenyl or alkyl substituted)*. Thiophene has a lower aromatic stabilization energy compared to indole and the replacement of one indole for a thiophene should allow for higher photostationary states to be achieved.

4. *Internal methyl groups*. The presence of methyl groups decreases the likelihood of photofatigue, by preventing oxidation aromatic carbazole.

This section features both phenyl and aliphatic substituted thiophene derivatives. Starting with the phenyl thiophene, since it can be prepared relatively easily. Additionally, the photoresponsive behaviour is likely more favourable for the phenylthiophene derivatives. The aryl group increases the tendency for the inhibitor to adopt the antiparallel structure in solution, favouring photoswitching.
Inhibitor targets featured in this section:

![Image of inhibitor targets]

**Figure 2.18** – Summary of the type 1 inhibitors – heteroaryl BIM analogues prepared. Targets include the phenylthiophene (A) and an alkylthiophene (B) series.

A decrease in activity may accompany the replacement of one of the two indole groups with a thiophene. In an early report by Davis *et al.*, 3- and 2-thienyl maleimides were synthesized and assayed, along with a variety of other heteroaryl derivatives to explore the structure activity relationships for the BIM derivatives. The reported IC$_{50}$ values for these compounds were 13.8 ± 3.9 µM and 5.2 ± 2.2 µM respectively.$^{68a}$

**Figure 2.19** – Structures of substituted thienyl maleimides.

The thiophene moiety does not offer any beneficial effects for the inhibitor design, and there is a limited amount of literature is available for its behaviour as a PKC inhibitor. The goal of this work however, is not to prepare a lead drug candidate, that exceeds potency and selectivity of existing inhibitors. Rather, it is to assess the effect of the inhibitory activity change with light induced isomerisation. We hypothesized that the
modification of the inhibitors with a thiophene group as a replacement for one of indole moieties would still permit binding to the ATP pocket, while allowing for enhanced photochemical performance, in comparison to bisindoles.

### 2.4.2 Binding conformation

In this inhibitor series, replacing one of indole groups with a phenylthiophene, is expected to change the binding ability with the active site. The structures of the ring-open and ring-closed isomers are shown in Figure 2.20 (a) below. The flexibility of the ring-closed isomer is restricted, and the end-to-end distance is predicted to be 12.7 Å. An overlap of this structure with the BIM scaffold, suggests that the size is too large to fit into the active site.

On the other hand, the two aryl groups of the ring-open isomer, can rotate to adopt a more compact conformation. When both aryl groups are flipped, relative to the maleimide bond, a shorter length of 7-9.1 Å is possible. In this case, superposition of the flipped structure of 2.6-o, with a BIM derivative in the active site of PKC, gives a conformation where the phenyl group extends out of the binding pocket to avoid steric clash with the enzyme, as shown in Figure 2.20 (b). Ideally, the changes in the end-to-end distance, will lead to measurable differences in binding affinity and enzyme activity.
Figure 2.20 – (a) Comparison of the 3-D structures of 2.6-o and 2.6-c. (b) Possible binding mode for the phenylthiophene derivatives series. Both the indole and the phenylthiophene groups are in a flipped position relative to the maleimide. (Image prepared using Pymol and PKC βII from PDB ID: 2I0E).

2.4.3 Type 1A Phenyl derivatives

Several synthetic routes have been developed for the construction of bisindolylmaleimide core and other related bisarylmaleimides derivatives. The retrosynthetic pathways for construction of the bisindolylmaleimide core are displayed below in Scheme 2.1. Generally, these can be divided into two common strategies, involving either (1) coupling reaction between an aryl group to a halo substituted maleimide, or (2) glyoxalate ester and amide condensation.
2.4.3.1 Route 1 – Faul condensation

Two distinct routes were considered and investigated for the synthesis of the phenylthiophene inhibitor targets. The first route shown in Scheme 2.2, described by Faul *et al.*,\textsuperscript{95} utilizes a glyoxalate ester condensation, as the key step for the construction of the BIM core. The reaction involves treatment of a THF solution of the indolylacetamide and the indolyl glyoxalate with potassium \( t \)-butoxide, at \( 0^\circ \text{C} \). Initially, the acetamide reacts with the glyoxalate ester to form the condensation product, which then undergoes a rapid intramolecular nucleophilic attack to give the succinimide intermediate. In the final step, the succinimide undergoes dehydration, creating the central double bond, to give the target maleimide in excellent yields.

**Figure 2.21** – Retrosynthetic analysis of the basic bisindolylmaleimide core, highlighting the key building blocks used in routes by published by Steglich,\textsuperscript{93} Lown\textsuperscript{94} and Faul.\textsuperscript{95}
Scheme 2.3 – Proposed mechanism for formation of the bisindolylmaleimide core, using the method reported by Faul et al. The key step involves condensation of an indole acetamide and indole-3-glyoxalate in the presence of potassium t-butoxide, followed by hydrolysis.96

Though this route was originally reported for synthesis of bis-indole compounds, later reports, detailed by Faul,96 and Gribble97 extend the synthesis to a range of asymmetric systems, with various heterocyclic moieties and combinations of starting materials.

2.4.3.2 Retrosynthetic analysis:

Figure 2.22 – Key intermediates envisioned for construction of the target bisarylmaleimide core of compound 2.4.
This route involves reaction of the indole-3-glyoxalate with the thienyl acetamide. Though alternatively, a thienyl ester and an indolyl glyoxamide combination could also be used to prepare the target BIM core. Literature procedures describe the latter route requiring several equivalents (at least 3.3 equiv) of the thiophene ester, to consume the glyoxamide, which otherwise, hinders the purification process.97

2.4.3.2.1 Synthesis of key intermediate 2.13

The preparation of the indole-3-glyoxalate is well known and several detailed procedures of its synthesis are reported in literature.98 Here it was prepared through reaction of commercially available 2-methyl indole with oxalyl chloride followed by reaction with sodium methoxide.

![Scheme 2.4 – Preparation of the indolyl glyoxalate 2.13.](image)

2.4.3.2.2 Synthesis of key intermediate 2.17

The first route attempted for thiophene acetamide preparation (outlined in Scheme 2.5) involved reaction of 2-methyl-5-phenylthiophene with sulfur and morpholine using a Willgerodt-Kindler reaction.99 We imagined that the thioamide product, 2.15 could be hydrolyzed to give the corresponding acid 2.16, and then finally the amide after reaction with ammonium hydroxide. Yielding the desired thiophene acetamide 2.17, in three steps. However, after conducting several trials, the first step proved to be problematic, and resulted in complex reaction mixtures of insoluble products. Compound 2.15 was not isolated and this route was abandoned.
To circumvent this problem, an alternative route was proposed for the synthesis of the acetamide based on a synthetic procedure that was used for another unrelated project. Though the route is more tedious, in terms of number of steps required, the route has been proven to work and the desired acid 2.23 can be isolated without any issues. Starting with 3,5-dibromo-2-methylthiophene, the first step of the synthesis involves a Pd(0) catalyzed cross coupling to install the phenyl group to the 5-position (not shown). Next 3-bromo-2-methyl-5-phenylthiophene, 2.18 was treated with n-butyllithium and quenched with DMF, followed by an acidic workup to give the corresponding aldehyde 2.19 in good yield. The aldehyde was reduced in the next step to an alcohol using sodium borohydride to give 2.20. The alcohol isolated after the reduction was used directly in the next step without further purification. Treatment with POBr₃ yielded the brominated derivative 2.21. The bromine was displaced using NaCN to give a cyano substituted derivative 2.22, this was later hydrolyzed to the acid 2.23 and then converted to the desired acetamide 2.17.

**Scheme 2.5** – Proposed synthetic route to prepare thiophene piece 2.17 using a Willgerodt-Kindler reaction.

**Scheme 2.6** – Alternative route for the preparation of thiophene acetamide piece, 2.17.
After isolation of the thiophene acetamide 2.17, the condensation step to prepare the BIM core was setup. One equivalent of both the indolylglyoxlate 2.13 and thiophene acetamide 2.17 were dissolved in THF. The solution was cooled to 0 °C and then treated with a 1 M potassium tert-butoxide solution and stirred at that temperature for 1 hr before allowing the reaction to warm to room temperature. After quenching with aqueous HCl, the reaction was heated to reflux, to induce the dehydration and generate corresponding maleimide final product 2.4. The product was isolated and purified, giving a bright orange coloured solid, in 54% yield.

2.4.3.2.3 Synthesis of bisarylmaleimide core:

![Chemical Structure](image)

Scheme 2.7 - Route 1 synthesis of phenylthiophene indolylmaleimide 2.4 using Faul’s condensation method.⁹⁶

2.4.3.3 Route 2 - Synthesis of phenylthiophene indolylmaleimide using Pd(0) Cross Coupling

The synthetic route outlined in the last section, involving condensation of the thienylacetamide with the indolyl glyoxalate ester, gave the desired indolyl-BIM product 2.4. However, the yield was satisfactory and required a tedious route to obtain the thiophene intermediate, which did not lend itself well to modification. We envisioned that a second, alternative route with fewer steps, could also be employed for the synthesis of the desired phenyl indolylmaleimide target. The second synthetic route involves a Pd catalyzed cross coupling reaction between a dihalomaleimide and boronic acid.¹⁰²,¹⁰³ The thiophene and indolylmaleimide intermediates were synthesized according to Scheme 2.8 and 2.9 respectively.
2.4.3.3.1 Synthesis of key intermediate 2.18:

The thiophene intermediate was prepared in one step using a Suzuki coupling. Despite having two reactive sites, where the phenyl substituent could be appended to, the material isolated gave the desired product, 2.24 in good yield.

![Scheme 2.8](image)

Scheme 2.8 – The thiophene piece was prepared in one step from the known compound 3,5-dibromo-2-methylthiophene 2.18 in one step using a Suzuki coupling.

2.4.3.3.2 Synthesis of key intermediate indolylmaleimide 2.29

The indole intermediate was prepared efficiently in two steps using a Grignard-type reaction, reported by Steglich et al.\textsuperscript{93} This reaction involves treatment of 2-methylindole with a solution of methyl magnesium bromide, followed by slow addition of maleimide 2.27. Using THF as a solvent, gives the mono-substituted indolylmaleimide. Switching the solvent to toluene, gives the bisindole product. The 2,3-dibromopyrrole is commercially available for \(~ $98 / 1g\), however can be prepared in a one pot procedure from N-methylpyrrole and NBS, followed by oxidation with nitric acid.\textsuperscript{94}
**Scheme 2.9** – Preparation of the indolylmaleimide precursor 2.29 from commercially available N-methylpyrrole, 2.26.93,104

**2.4.3.3 Coupling reaction to form bisarylmaleimide core:**

The target bisarylmaleimide was prepared using a convenient cross coupling reaction between the two key intermediates. Initially, the phenylthiophene bromide is treated with n-BuLi, undergoing a lithium halogen exchange reaction, and then subsequently quenching the resulting species with tributyl borate, to give the corresponding boronate ester. Without isolation, the boronate ester is cannulated into the reaction mixture containing the bromoindolylmaleimide 2.29 and Pd(0). The product 2.30 was isolated from the reaction mixture, and purified using column chromatography to yield a bright orange coloured solid. Though yields for this reaction were moderate, the one pot approach to construct the BIM core of 2.30 is significantly more efficient than the previous method, utilizing the acetamide derivative. Therefore, leading to a much shorter and convenient synthesis.

\[
\begin{align*}
\text{Br} & \quad 1) \text{n-BuLi, THF, -60ºC} \\
& \quad 2) \text{tributyl borate} \\
& \quad 3) \text{2.29, Pd(PPh_3)_4, K_2CO_3} \\
& \quad \text{DME/H_2O, } \Delta \\
\text{2.18} & \quad \rightarrow \\
& \quad 65\% \\
\text{KOH, EtOH, } \Delta \\
\text{2.30} & \quad \rightarrow \\
& \quad 90\%
\end{align*}
\]

\[
\begin{align*}
\text{2.31} & \quad \text{NH_4OAc, CH_3CO_2H, } \Delta \\
& \quad \rightarrow \\
& \quad 84\%
\end{align*}
\]

\[
\begin{align*}
\text{2.6} & \quad \text{N-H maleimide} \\
& \quad \rightarrow \\
& \quad 84\%
\end{align*}
\]

**Scheme 2.10** – Synthesis of the target structure using a Suzuki coupling to construct the BIM core 2.30, and followed by a subsequent hydrolysis reaction to give the anhydride 2.31 and an ammonolysis to yield 2.6.

The last steps involved in the synthesis, to convert the N-methyl maleimide 2.30 to the desired N-H maleimide 2.6, are relatively simple to conduct and high yielding. Initially, 2.30 is heated in an aqueous ethanol and KOH mixture, to generate the anhydride 2.31. In the final step, the anhydride is converted to the maleimide by refluxing in ammonium
acetate and acetic acid. After cooling, 2.6 conveniently precipitates from the reaction mixture, the orange solid is collected and washed, yielding pure 2.6. Overall, this second synthesis route for the preparation of the phenyl thiophene indolylmaleimide derivatives, proved to be more efficient. Less overall steps were necessary, and the indolylmaleimide precursor 2.29, could be prepared on a large scale and coupled to various different phenyl thiophene derivatives in one step.

2.4.3.3.4 Synthesis of the methoxy and dimethylamino derivatives

The above procedure was also used for the preparation of the methoxy, 2.7 and dimethylamino, 2.8 derivatives. Their syntheses are outlined in Scheme 2.11 below. Conveniently, indolylmaleimide 2.29, that was used for the previous synthesis of 2.6 was also used in these steps. The thiophene precursors, 2.32 and 2.33, were prepared using a Suzuki coupling between 2.24 and the appropriate boronic acid, (see experimental for synthesis of these precursors).

![Scheme 2.11](image)

**Scheme 2.11** – General synthesis of methoxy 2.7 and dimethylamino 2.8 derivatives using Pd(0) catalyzed cross-coupling.

During the coupling reaction to produce the dimethylamino derivative, 2.8 the desired product precipitated out of the reaction mixture, requiring a simple wash step with DCM to yield the pure product.

2.4.3.3.5 Synthesis of the demethylated derivative

The demethylated indole derivative 2.5 was prepared using the same methodology as used earlier, for the synthesis of compounds 2.6, 2.7 and 2.8. The same conditions as described earlier for the preparation of the bromo-indolylmaleimide 2.29 were also used.
here to construct the demethylated version, 2.35. Although indole was used as a starting material instead of 2-methylindole.

Scheme 2.12 – Synthesis of the demethylated indole phenyl thiophene 2.5. The indolylmaleimide can be prepared by treatment of indole with methyl magnesium bromide, followed by N-methyl dibromomaleimide (right). The bisarylmaleimide core can be constructed using a Pd(0) coupling. The N-methylmaleimide is converted to the anhydride and then to the desired N-H maleimide (left).

Phenyl thiophene 2.18, was treated with n-BuLi, the resulting anion was quenched with tributyl borate and used directly in the Pd catalyzed cross-coupling reaction. The resulting product was a yellow colour, in contrast to the 2-methylated derivatives prepared, 2.4, 2.6, and 2.7 were all bright orange, and 2.8 was deep red. The absence of the 2-methyl group in 2.5, reduces steric hindrance and allows for more rotational freedom. The yellow colour suggests less conjugation between the maleimide and indole.

### 2.4.4 Photoresponsive performance and characterization of phenyl derivatives

The photochemical performance of the synthesized compounds were investigated using a combination of UV-visible absorption spectroscopy, fluorescence emission spectroscopy, and NMR spectroscopy. The light induced changes in structure can be monitored using any of the above techniques, however UV-visible absorption spectroscopy is often the most convenient, since it provides a relatively quick method to assess changes induced by light and requires very little material.
All ring-closing experiments were conducted in solution, and using one of the following light sources: a hand-held lamp for visualizing TLC plates, with an emission centered at 365 nm or 312 nm (Spectroline E-series) or using the light from a PTI Quantamaster Spectrofluorimeter, capable of producing monochromatic light (typically 450 nm light, 10 nm slit width, ~1 mW/cm², unless otherwise stated). In most cases, the compounds investigated in this section, undergo light induced ring-closure in the presence of both light sources, however the yields of the resulting ring-closed isomer, differ with irradiation wavelength. The bisarylmaleimide scaffold absorbs in the visible region, and monochromatic visible light from the spectrofluorimeter was found to be an ideal light source for ring-closing experiments. However, the hand-held TLC lamp was also used for some experiments, due to its convenience.

A tungsten light source, with a >490 nm cut-off filter (Elmo Omnigraphic 300 AF, 37 mW/cm²) was used for ring-opening experiments.

### 2.4.4.1 UV-visible absorption properties of series 1 inhibitor scaffold

#### 2.4.4.1.1 Photochemical ring-closure

Irradiation of a solution of **2.6-o** in THF, 50 µM with 450 nm light (spectrofluorimeter source, 10 nm slit width) produces the ring-closed isomer **2.6-c**. The ring-closure process was monitored by UV-visible absorption spectroscopy, see Figure 2.23 (b). A small decrease in the intensity of the absorption band at 440 nm from **2.6-o** is observed, which is accompanied by the appearance of a new broad absorption band centered at 568 nm, and a second narrower band at 407 nm from **2.6-c**. The ring-closure process generates an extended π-conjugated backbone, which results in a red-shifted absorption spectrum. A visible colour change also takes place, the solution changes from pale yellow to pink/purple, as **2.6-c** is formed. The solution was irradiated for a total of 8 minutes, after which point, the photostationary state was reached and no further changes in the absorbance spectrum were observed with continued irradiation. These observed absorption changes are consistent with what is typically observed for photoresponsive diarylethenes. The coloured ring-closed isomer remains stable in the dark, with no detectable cycloreversion after 24 hours.
Figure 2.23 – (a) Irradiation of a THF solution of 2.6-o with 450 nm light, produces the ring-closed isomer 2.6-c. (b) The UV-visible absorption spectra of 2.6-o in THF (c = 50 µM), upon sequential (60 s intervals) of irradiation with 450 nm light. (c) Changes in the absorption band at 570 nm, as 2.6-c is generated. The changes in the absorbance plateau as the photostationary state is reached.

The table below summarizes the absorption maxima, and the corresponding extinction coefficients for the ring-open isomer and photostationary states obtained for 2.4-2.8. As expected, the UV-visible absorption spectra for most of the compounds in the series, share a number of similar features, displaying bands at ~430-440 nm and 275-285 nm. With the exception of 2.5, where the absorption band is shifted to 420 nm. The removal of the methyl group at the 2-position on the indole ring, had the most dramatic influence on the appearance of the spectrum, likely reflecting the molecules rotational flexibility and reduced co-planarity. The ring-closed isomers for 2.6-2.8 have a similar absorption maxima in the visible region, giving a broad peak at ~560-570 nm. The peak for the N-methylated derivative, 2.4 is red shifted, and appears at 580 nm.
Table 2.5 – Summary of UV-visible absorption properties of 2.4-2.8

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ring-open</th>
<th>Photostationary state</th>
<th>Conversion yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\lambda_{\text{max}}$ / nm ($\epsilon$ / L mol$^{-1}$cm$^{-1}$)</td>
<td>$\lambda_{\text{max}}$ / nm</td>
<td>ro $\rightarrow$ rc (%)</td>
</tr>
<tr>
<td>2.4</td>
<td>440 (6715), 280 (23136)</td>
<td>580, 421, 288</td>
<td>38$^a$</td>
</tr>
<tr>
<td>2.5</td>
<td>420 (6935), 276 (21738)</td>
<td>$\sim$550, $\sim$410, $\sim$275</td>
<td>$&lt;5^b$</td>
</tr>
<tr>
<td>2.6</td>
<td>437 (5976), 277 (22041)</td>
<td>568, 407, 287</td>
<td>35$^a$</td>
</tr>
<tr>
<td>2.7</td>
<td>432 (8974), 277 (32780)</td>
<td>557, 401, 312, 270</td>
<td>N.M.</td>
</tr>
<tr>
<td>2.8</td>
<td>435 (5203), 325 (19880)</td>
<td>564, 410, 326, 264</td>
<td>N.M</td>
</tr>
<tr>
<td></td>
<td>285 (17165)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: The measurements for each compound were performed at room temperature (~20 °C) and prepared in a solution of THF (c = 50 $\mu$M), with the exception for 2.8, which was prepared in a 9:1 mixture of hexanes/THF (c = 50 $\mu$M). $^a$The photoconversion yield for 2.4 and 2.6, was estimated from a $^1$H NMR of the photostationary state mixture. The photostationary state mixture was prepared by irradiating a concentrated solution (c = 1x10$^{-3}$ M, 10 mL) in THF, with 312 nm light for ~90 minutes, until the photostationary state was reached (progress was periodically checked by dilution of aliquots, and measurement of the UV-vis absorption spectrum). The solvent was removed under reduced pressure, dried in vacuo, and then redissolved in DMSO-$d_6$ for analysis (each of the steps mentioned were performed in the absence of light). $^b$The conversion yield was estimated to be low due to the very small changes in the absorption spectrum with irradiation. The abbreviation N.M. = not measured.

The photostationary state of compound 2.4 was estimated to be $\sim$38% using $^1$H NMR. This indicates that the light induced conversion of 2.4-o to 2.4-c is not complete, and a significant amount of the 2.4-o isomer remains in the photostationary state mixture after irradiation. 2.6-o was later estimated to be $\sim$35%. The photostationary states of the remaining compounds in the series were not measured by $^1$H NMR. However, based on their structural similarities and absorption spectra before and after light irradiation, the extent of ring-closure was estimated to be similar for compounds 2.4, 2.6 and 2.7 (it is important to note that these comparisons are qualitative).

The dimethylamino derivative 2.8-o was particularly interesting and exhibited the largest changes in its UV-visible absorption spectrum with light irradiation compared to the other derivatives in the series, see Figure 2.24 (b). The difference in behaviour can likely be attributed to the $p$-dimethylamino donor group, appended to thiophene. However, whether the more dramatic spectral changes with light irradiation, are due to a higher photostationary state, or are from a higher molar extinction coefficient for 2.8-c,
was not confirmed. Attempts to isolate \textbf{2.8-pss} for NMR analysis were made, however a number of obstacles were met. The ring-closure process for \textbf{2.8-o} is very sensitive to solvent polarity, and only proceeds to a significant extent in hydrocarbon based solvents (i.e. hexanes). See next section for results and discussion of the solvent dependence of the phenylthiophene series. Additionally, solubility of \textbf{2.8-o} (and likely \textbf{2.8-c}) are moderate in hexanes. The relatively dilute concentrations, used for UV-visible measurements, \(~50 \mu M\) were sufficient to dissolve \textbf{2.8}. However, at increased concentrations required for isolation and NMR analysis, \(~1000 \mu M\), significant precipitation takes place, and the photostationary state was not determined.

![Figure 2.24](image)

**Figure 2.24** – (a) Irradiation of a hexanes solution of \textbf{2.8-o} with 450 nm light, produces the ring-closed isomer \textbf{2.8-c}. (b) The UV-visible absorption spectra of \textbf{2.8-o} dissolved in THF (\(c = 50 \mu M\)), upon sequential (60 s intervals) irradiation with 450 nm light. (c) Changes in the absorption band at 570 nm, as \textbf{2.8-c} is generated. The changes in the absorbance plateau, as the photostationary state is reached after \(~8\) min total light exposure.
2.4.4.1.2 Influence of 2-methyl groups

An investigation of how the methylation at the 2-position of indole effects photochemical behaviour was also included. Traditionally, the methyl groups or another alkyl substituent are added to both of the internal groups of the DTE thiophene to reduce the occurrence of oxidative aromatization after ring-closure and enable reversibility. In the absence of substituents on both of the aryl groups, as shown in the scheme below, the DTE can undergo UV induced cyclization, followed by deprotonation to generate the planar fully aromatic species.\(^{20}\) The latter process is irreversible.

![Scheme 2.13](image)

**Scheme 2.13** – In the absence of substituents placed onto the internal positions of the thiophene rings, the ring-closed isomer can undergo irreversible conversion to the fully aromatized structure.

Presumably, removal of one of the two methyl groups would allow for photoswitching, without full oxidation to the fully aromatized structure. However, the absence of a methyl group at the 2-position on indole lead to a reduction in the photocyclization yield. The figure below includes an example of the changes in the absorption spectrum before and after exposure to 440 nm light. Although, some conversion appears to have taken place, as a very low intensity peak appears centered at \(~550\) nm is visible in the absorption spectrum after 440 nm light irradiation. The resulting changes in the absorbance spectrum of ring-open isomer, \textbf{2.5-o} with exposure to light were very small, indicating that the isomerization process has not taken place to much extent.
Figure 2.25— (a) Irradiation of a THF solution of 2.5-o with 440 nm light, produces the ring-closed isomer 2.5-c. (b) The UV-visible absorption spectra of 2.5-o dissolved in THF (c = 50 µM), before and after 480 s of 440 nm light. (c) Changes in the absorption band at 550 nm, as 2.5-c is generated.

The results from the studies in this section indicate that removal of one of the 2-methyl substituents leads to a significant decrease in photostationary state yield. The additional steric bulk from the methyl group could limit the degree of rotation possible for the aryl groups and increase the percentage of molecules in the antiparallel conformation. Substitution at the 2,2'-position is therefore important for maintaining a moderate photoconversion yield in this derivative series.

2.4.4.1.3 Solvent dependent photostationary state

During the course of UV-visible experiments, the photoinduced ring-closure process was proven to be strongly dependent on solvent, and the measured cyclization yields vary considerably depending on the solvent medium chosen. Unfortunately, the low photocyclization yields in polar solvents, prevents the demonstration of a fully
reversible photoresponsive inhibitor in enzyme studies (which require an aqueous based solvent). Therefore, the in situ ‘on’ and ‘off’ cycling of the inhibitory activity is not possible, since the deactivation of the active photoisomer (conversion of the ring-open isomer to the ring-closed isomer) does not take place to much extent in aqueous media. However, these findings dictate that a one-way activation process is still possible. The activation process (cycloreversion) can be carried out efficiently in all solvents, including the aqueous media used for biochemical studies. The ring-opening process is not only solvent independent, but also takes place rapidly (described further in the next paragraphs), with less damaging visible light, avoiding the need for long irradiation times. This feature could therefore, be an improvement over existing inhibitor systems based on photocages and existing diarylethene photoswitches, which often require ultraviolet light and longer irradiation times.\textsuperscript{105}

With concern about the performance of the photoresponsive inhibitor candidates in biochemical assays, the solvent dependent behaviour was investigated further. To measure the variation in photochemical behaviour, solutions of compounds \textbf{2.6-o} were prepared in solvents of differing polarities, and the changes in their absorption spectrum with 450 nm light irradiation was monitored over time, see UV-visible traces below in Figure 2.26 for comparison photoisomerization extent in hexanes, THF and DMSO. There is a small but noticeable red shift with increasing solvent polarity, $\lambda_{\text{max}}$ (hexanes) = 433 nm, $\lambda_{\text{max}}$ (THF) = 442 nm, and $\lambda_{\text{max}}$ (DMSO) = 446 nm suggesting the molecule is somewhat sensitive to polarity. The absorption spectrum before and after irradiation in THF and hexanes, appears relatively similar for \textbf{2.6-o}, suggesting that the photocyclization yield in these two solvents is comparable. However, when changing the solvent to DMSO, the photocyclization reaction is strongly suppressed, and only very small changes in the absorption spectrum are visible at all, see inset of Figure 2.26 (c), for a zoom in of the changes in absorbance 550-650 nm. Other wavelengths, power intensities, and irradiation times were also tested, however, this did not achieve the similar change in absorption spectrum over time that was observed in THF (or related non-polar solvents), instead prolonged irradiation results in degradation to the compound.
**Figure 2.26** – UV-visible absorption spectrum of before (2.6-o) and after (2.6-PSS) irradiation with 450 nm light (fluorimeter source, 10 nm slit width, for ~8 minutes) in solvents of differing polarity (a) hexanes (0.5% THF), (b) THF (100%) (c) DMSO (0.5% THF). All solutions are 50 µM, prepared from the same stock solution in THF.

This same pattern of solvent dependence was observed for the remaining derivatives prepared in the series. A summary of the absorption changes at the $\lambda_{\text{max}}$ for the ring-closed isomer 2.4-c, when 2.4-o is irradiated with 312 nm light in various solvents is given in **Figure 2.27a** (irradiation source, hand-held UV-lamp). Interestingly, there appears to be a point in the solvent polarity where the extent of ring-closure drops quickly. Literature findings equate the dielectric constant, as the predominant factor influencing the photostationary state yield.\textsuperscript{91d,e} An increase in the dielectric constant of the solvent medium, appears to result in a decrease in the quantum yield, and the photostationary state of the compound. However, the dielectric constant alone, is likely not the only solvent parameter that influences the conversion yield.
Figure 2.27 – (a) Changes in the UV-visible absorption spectrum at the $\lambda_{\text{max}}$ for ring-closed isomer 2.4-c, when 2.4-o is sequentially irradiated in with 312 nm light, in various solvents. The change in absorbance provides an estimate of the extent of photoconversion. All measurements were prepared from a stock solution in THF, and the final concentration used for measurements was 50 $\mu$M. (b) Changes in the UV-visible absorption spectrum at the $\lambda_{\text{max}}$ for ring-closed isomer 2.4-c (at the photostationary state) when irradiated with >490 nm light.

Reports by Irie et al, detail similar findings with thiophene and benzothiophene anhydrides or maleimides, that exhibit strong solvent dependent photoswitching behaviour.$^{91d}$ Low quantum yields were reported for acetonitrile, in comparison to hexanes, benzene or THF. The poor photoisomerization yield could be due to a number of reasons. A commonly described theory for the solvent dependent photoconversion yields observed for bisarylmaleimides and bisarylanhydrides, is the presence of a twisted intramolecular charge transfer state (TICT).$^{91c-e}$ In solution, the aryl groups are free to rotate, and can adopt a number of conformations. Photocyclization occurs from the antiparallel, planar conformation, shown in Figure 2.28 (right). Here, the distance between the two aryl groups is close enough for bond formation. In polar solvents, the molecule rearranges, and the twisted structure predominates due to the strong charge
The indole is rotated perpendicular to the maleimide group, and photocyclization is not possible, **Figure 2.28** (left). Therefore after excitation in polar solvents, relaxation to the ground state occurs, without photoswitching to the ring-closed isomer. Reports that feature substituted phenylthiophene maleimides, detail that the photostationary state in polar solvents, DMSO is nearly quantitative.\textsuperscript{91a} The sensitivity of a compound to solvent polarity is dependent on steric and electronic features.

**Figure 2.28** – In the twisted form of 2.6-o, (predominant in polar solvents), the indole and the phenylthiophene groups are perpendicular to one another. This conformation does not allow for photocyclization to take place, and the excited state returns to the ground state without formation of the ring-closed isomer 2.6-c. Whereas in the planar form of 2.6-o, the pendant groups are positioned at an ideal orientation for cyclization to occur from the excited state. Illustration for figure inspired by diagram by Irie et al.\textsuperscript{91e}

In all cases, exposure of the ring-closed isomers or photostationary state mixtures of the phenylthiophene derivatives with light passed through a $>490$ nm cut-off filter, leads to nearly quantitative regeneration of the open isomers, indicating the full reversibility of the closed to open photoisomerization. Kinetics for the ring-opening of 2.4–PSS (photostationary state mixture) are presented in solvents of different polarities in **Figure 2.27** (b). Unlike the ring-closing reaction, which was shown to be highly dependent on photostationary state, the rate of ring-opening process is very similar in the solvents tested (under these irradiation conditions), as the traces for DMSO, hexanes, CH$_3$CN and THF all appear to overlap. The cycloreversion rates for compound 2.4, are therefore not significantly dependent on solvent medium. The timescale for ring-opening is relatively fast, compared to traditional diarylethene derivatives. Nearly 95%
conversion takes place in 4 seconds. This is likely caused by the higher aromatization energy of the indole group compared to other heterocycles, for example thiophene. Exposure of the ring-closed isomer or photostationary state mixtures to ambient lab light, also induces ring-opening on the order of a few minutes (or within a few seconds for dilute solutions). The ring-closed isomers are however, stable in the dark at room temperature, for at least several months.

The effects of solvent polarity are most pronounced in the dimethylamino derivative 2.8. Unlike for hexanes, when the photoreaction was performed in THF, there were no discernable changes in the absorbance spectrum. The intense band at 570 nm that was observed immediately after exposure to 2.8-o to blue light in hexanes solution was not present in THF, and was very weak in dioxane even after prolonged exposure. The other compounds in the phenylthiophene series, exhibited good photochromism in THF, and the suppression of conversion yields, did not appear until the solvent polarity was increased further. The difference between 2.8 and the remaining compounds in phenyl thiophene series (2.4-2.7), is the p-dimethylamino group which appeared to be the source of the large deleterious effects on the photoconversion. Mixtures of THF and hexanes were also investigated (data not shown), up to 25% THF/hexanes yielded comparable conversion yields. Although at higher percentages of THF, this led to negligible conversion yields. The dimethylamino derivative is therefore is more sensitive to polarity.
2.4.4.1.4 Wavelength dependence on photostationary state

The optimal wavelength for conversion depends on the absorption spectrum of the indolylmaleimide derivative. This was found to vary slightly with photoswitch structure, although, generally the highest photostationary state was achieved with 440-455 nm light. An example is shown below in Figure 2.30 of how the irradiation wavelength influences the photostationary state, for 2.6. THF solution (c = 50 µM) of 2.6-o was irradiated with light of different wavelengths for a total of 600 seconds each to ensure the photostationary state is reached. The absorption at the $\lambda_{\text{max}} = 570$ nm (representative of 2.6-c), achieved after the photostationary state, is shown for each irradiation wavelength. From the data, the optimal conversion occurs with the 450 nm source, with the largest change in absorbance measured after the allotted irradiation time period.
Figure 2.30 – Summary of the results obtained from experiments investigating the influence of irradiation wavelength on the photostationary state for compound 2.6. Stirred THF solutions of 2.6-o (50 µM) were irradiated for a total of 600 seconds in a quartz cuvette (fluorimeter source, 10 nm slit width) to ensure the photostationary state is reached. The bars in the figure correspond to the absorbance value of 2.6-c ($\lambda_{\text{max}} = 570$ nm) at the photostationary state achieved with the different irradiation wavelengths. The optimal wavelength for maximal conversion is with ~450 nm.

The majority of phenylthiophene bisindolylmaleimides synthesized in this series, display a significant improvement over traditional bisindolylmaleimides in terms of photoswitching ability. This is evident from the larger extent of change to the UV-visible absorption spectrum that occur with light irradiation. An exception was observed for the non-methylated indole derivative, which despite irradiation with different wavelengths of light, did not undergo photocyclization to much extent. Internal substitution of the pendant aryl groups, with a methyl group (or substituent other than H) is considered necessary for enhancing the photoswitching behaviour. The presence of the methyl groups limits the degree of rotation possible for the aryl groups. Additionally, the conjugated indolylmaleimide structure, shifts the absorption spectrum to the visible region, thus allowing for both the cyclization and cycloreversion processes to take place using visible light; an advantage of traditional diarylethene systems that require UV light for the ring-closure process. An investigation of the optimal wavelength of light to be used for cyclization was also conducted, and found that 450 nm is typically most ideal, though longer wavelengths, up to 475 nm can also be utilized.
2.4.4.2 *Fluorescence emission spectroscopy*

2.4.4.2.1 Solvent dependent emission

The indolylmaleimide derivatives have an intense emission bands at 525-575 nm in solvents that are non-polar. The absorption spectrum of the ring-open isomer does not shift significantly when solvent polarity is increased (<20 nm as shown for compound 2.6), although, the emission spectrum is very sensitive to the local solvent environment surrounding the compound. The change in the emission wavelength with solvent polarity was measured for compound 2.6-o, see Figure 2.31 (a-b) below for a comparison of the absorption and emission in a range of solvents. There is also a dramatic change in the emission peak shape and peak intensity with changing solvent. Compound 2.6-o showed an intense, structured emission band at 525 nm in hexanes. Whereas in solvents of moderate to high polarity show much weaker and broadened emission bands.

Fluorescence can be used as an indicator of the sensitivity of the compound to solvent polarity and provide information about the nature of the excited state. A Lippert-Mataga plot was used to evaluate the sensitivity of a compound’s excited state to solvent polarity. The Stokes shift ($\Delta \nu$, cm$^{-1}$) in several different solvents was measured, and the obtained values were plotted as a function of the solvent orientation polarizability ($\Delta f$) as defined in Figure 2.31 (c). The solvent orientation polarizability is a parameter that takes into account the solvent’s dielectric constant ($\varepsilon$) and refractive index ($n$), and is a measure of the solvent’s dipole moment and the mobility of electrons in the solvent. A linear increase in the Stokes shift with an increase in solvent polarity was observed which suggests that the excited state has a larger dipole moment than the ground state, see Figure 2.31 (c).
Molecules that contain an electron donor group (i.e. indole, thiophene, benzothiophene) conjugated to strong electron acceptor group (for example maleimide and anhydride) can exhibit charge separation, which can influence the orientation of the functional groups in the molecule. This concept was mentioned in the previous section on the solvent dependent photostationary state. For photoresponsive molecules, the consequence of the charge separation, is that it can shift the equilibrium between the planar and perpendicular states of the ring-open isomer, refer to Figure 2.28 in the previous section. Therefore based on the slope of the plot in Figure 2.31 (c) and the highly solvent dependent photocyclization yields, the phenylthiophene derivatives
presented in this series, likely adopt a perpendicular conformation in polar solvents. Unfortunately, this hinders photoswitching.  

2.4.4.2.2 Using Fluorescence emission to monitor photocyclization progress

The fluorescence emission intensity of the indolylmaleimide derivatives can be modulated with light. This provides an alternative method to monitor the ring-closure process. Excitation with the wavelength used to induce ring-closure, ~450 nm, 2.6-o exhibits an intense and broad fluorescence band centered at ~525 nm in hexanes solution. This excitation wavelength also induces ring-closure, therefore converting 2.6-o to 2.6-c. There is a prominent decrease in the measured emission intensity with prolonged light exposure. This could be due to the decrease in the concentration of the ring-open isomer as it is converted to the ring-closed form. Additionally, the ring-closed isomer 2.6-c has an absorption band in that overlaps with the emission band of the ring-open isomer 2.6-o. Therefore, energy transfer from the ring-open isomer to the ring-closed isomer is also possible leading to quenching of the fluorescence emission of the ring-open isomer to some extent. The progress of the ring-closure was monitored over time, and a decrease in the intensity of the emission at 525 nm, decreased until the photostationary state was reached.
Figure 2.32 – (a) Overlap of the fluorescence emission spectra for 2.6-o and 2.6-PSS (12.5 µM, hexanes, λ_{ex} = 450 nm, 2 nm slit width). (b) The decrease in emission intensity at 525 nm of compound 2.6-o over time, as it exposed to 450 nm light, forming the ring-closed isomer 2.6-c.

2.4.5 Enzyme activity

After characterizing the photoisomerization behaviour of the phenylthiophene series compounds 2.4-2.8, we decided to investigate how the introduction of the phenylthiophene group, influences the enzyme activity and whether or not, light can be used to turn ‘on’ the inhibitory effects. Unfortunately, fully reversible conversion between the ring-open and ring-closed isomers would not be possible due to the suppressed ring-closure yields in polar solvents. However, we argued that the relatively fast ring-opening kinetics and the one way activation from the inactive state (presumably the ring-closed isomer) using visible light could still provide a convenient and unique system for light controlled enzyme activity.

2.4.5.1 Assay method

There are a number of methods available to evaluate protein kinase activity. Various assay formats and detection methods have been developed, ranging from radiometric, fluorescence intensity, fluorescence polarization, fluorescence resonance energy transfer (FRET), time-resolved fluorescence, time-resolved fluorescence resonance energy transfer (TR-FRET), and chemiluminescence. Despite the variety in detection methods, most rely on detection of either phosphate transfer to a substrate (quantifying the amount of phosphorylated product), or by quantification of the ratio of ATP to ADP during the course of the phosphorylation reaction.

To investigate the activity of the newly synthesized photoresponsive inhibitors in the phenylthiophene series, a commercially available colorimetric solid phase enzyme linked immunosorbant assay (ELISA) kit was initially selected for the assay method. This method was chosen for several reasons, it allows for a range of different PKC sources and samples types to be used in the assay, including cell lysates, tissue samples, and purified protein. Additionally, the reagents are simple to handle and store, and the readout can be measured easily (conventional UV-visible absorption plate reader).
brief description of the assay, a synthetic PKC pseudosubstrate pre-coated wells of a 96 well plate are incubated with the kinase, cofactors and either solutions of the inhibitors in DMSO, or DMSO as a negative control (the concentration of DMSO did not exceed 1%). The kinase phosphorylation reaction was initiated with ATP addition and allowed to proceed for 1 hour before stopping the reaction. A biotinylated polyclonal antibody that can recognize the phosphorylated peptide is added and incubated for 1 hour. The wells are then washed with buffer, to remove excess antibody. Afterwards, horseradish peroxidase bound to streptavidin was added to the wells, incubated for 1 hour, washed to remove the excess, and then lastly, treated with o-phenylenediamine substrate which produces a colour. The intensity of the colour is proportional to the enzyme activity.

Later on in the chapter, the assay method was changed to a homogeneous ratiometric FRET-peptide based assay format, with coupled enzyme detection system. This assay is more sensitive to contaminants, and therefore requires a purified PKC source. However, the homogeneous format is faster and more convenient to use. The details of this particular assay will be reviewed in the next section.

2.4.5.2 Assay development

Prior to setting up inhibitor screening of compounds 2.4-2.10, the quantity of protein kinase required was optimized to give useful readout under the assay conditions employed. The extent of phosphorylation within 1 hour at room temperature incubation was evaluated and used to determine an optimal amount kinase to use for with screening later on. PKC β-II, a classical PKC isoform was selected for studies, and its concentration was varied from 80 ng to 2.5 ng during the assay. The other assay parameters (i.e. the concentration of substrate, ATP, and lipid activators, Ca$^{2+}$, buffer concentration, reaction time, temperature, etc.) were kept constant in this section. A standard curve was created by plotting the optical density (OD) measured spectrophotometrically versus the concentration of PKC in the assay is shown below in Figure 2.33.
At high concentrations of kinase, the signal deviates from linearity. The curve shows saturation beyond 40 ng of PKC within the duration of the 1 hr assay. From the curve, ~2 to 25 ng PKC per assay gives a linear readout. A concentration of ~12.5 ng/assay was selected as a reasonable quantity of PKC to use in screening studies under these conditions.

2.4.5.3 Phenylthiophene derivative inhibitory activity screen

A preliminary in vitro screen was conducted to assess the inhibitory potency of the phenylthiophene compounds featured in the series. The ring-open isomers of 2.4, 2.6 and 2.7 along with the commercially available inhibitor, GF 109203X were assayed at a concentration of 10 µM. Inhibitor stock solutions were prepared in 100% DMSO and diluted in the assay buffer prior to use (DMSO concentration did not exceed 1%). The results of the screening are summarized in the table below.

Table 2.6 – Summary of activity for selected compounds of the phenylthiophene series against PKC

<table>
<thead>
<tr>
<th>Compound</th>
<th>Activity at 10 µM (% inhibition relative to DMSO control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.4-o</td>
<td>62</td>
</tr>
<tr>
<td>2.6-o</td>
<td>71</td>
</tr>
<tr>
<td>2.7-o</td>
<td>66</td>
</tr>
<tr>
<td>GF 109203X</td>
<td>94</td>
</tr>
</tbody>
</table>
The compounds in the series demonstrated moderate inhibition at this concentration. Compound 2.6-o showed the largest effect on the phosphorylation activity, inhibiting the kinase activity by 71% relative to the control (1% DMSO). These results are promising for the purposes of the light controlled inhibitor project. However, a clear decrease in the inhibitory activity accompanied the replacement of one indole groups with a phenylthiophene. The bisindolylmaleimide inhibitor, GF 109203X exhibited nearly complete abolishment of activity at the same concentration.

Based on these initial results, compound 2.6 was selected for further investigation. Several studies were conducted to evaluate the dose-response behaviour of 2.6-o and ring-closed isomer 2.6-c. Although, when expanding the assay to include more samples, there were large deviations in the data points. The assay was repeated multiple times to investigate the origin of the data fluctuations. It appeared that the results were dependent on the order the samples were prepared in. Samples were pre-diluted in buffer, prior to addition to the reaction mixture containing enzyme. The longer the incubation time in the aqueous mixture, the larger the fluctuations in the data. There was some concern that the inhibitor stability in the buffer system may have been limited since the diluted inhibitor solutions appeared cloudy after some time. The standard inhibitor GF 109203X was also included in the screening assay to serve as a comparison. In all cases, GF 109203X behaved as expected. The dose-response data collected for this compound yielded an IC\textsubscript{50} that closely matched literature values.\textsuperscript{47} In contrast to 2.6-o, GF 109203X did not show time dependent enzyme inhibition.

A literature search revealed that the strong dependence on the assay conditions (i.e. incubation time) were characteristics of aggregate induced inhibition.\textsuperscript{109} At micromolar concentrations, inhibitor candidates can form aggregate species in aqueous solution. Although the monomeric compound is not pharmacologically active, the oligomeric aggregates can form above a critical concentration that can inhibit enzyme activity by sequestering or adsorbing them onto their surface. The adsorbed enzyme has restricted access to substrate, reducing its ability to interact with substrates, cofactors and other functional molecules required for activity. The macromolecules are capable of inhibiting enzymes non-specifically and lead to false positives. This type of inhibition is typically not desired since it is non-specific and the inhibitor concentration is difficult to
control. Addition of BSA or non-ionic surfactants can help to solubilise the aggregate species. The assay was repeated, and the non-ionic surfactant, Brij-35 was added to the buffer (0.1%) prior to dilution of 2.6-o. The results from the assay are plotted in Figure 2.34, and no inhibition was measured at all concentrations tested for 2.6-o.

![Graph](image)

**Figure 2.34** - Log of the concentration (µM) of 2.6-o versus the percentage relative activity of the control sample, (1% DMSO). The buffer contains the surfactant Brij-35 to prevent formation of aggregates.

The initially promising results from the biochemical assay of compound 2.6-o were likely due to aggregate species. When 2.6-o is solubilised with addition of detergent to the assay buffer, the inhibitors appeared to have little effect on the activity of PKC. In summary, the bulky phenyl analogs display poor inhibitory activity compared to the bisindolylmaleimides. In conclusion the phenyl group is not an appropriate substitution for indole in the inhibitor design.

### 2.4.5.4 Solubility of phenyl derivatives

The observations from the inhibitor screening assays suggested that the unusual time dependent activity of the phenylthiophene compounds could be due to non-specific inhibition from small aggregate species formed in solution. The addition of non-ionic surfactant to solubilise the aggregating particles appeared to attenuate this effect. This section of the chapter describes a series of stability measurements of the phenylthiophene derivatives, 2.6-o and 2.6-c using a combination of UV-visible absorption spectroscopy and DLS to confirm the presence of aggregates. Additionally,
we hoped to identify a screening procedure to use with future compounds to identify potential aggregators prior to enzyme testing. The low solubility was not overtly obvious based on visual inspection of the aqueous solutions of the compounds, hence the need for a reliable stability screening procedure.

UV-visible absorption spectroscopy is useful for predicting changes to the concentration of the phenylthiophene series. A stable solution should not exhibit significant changes in absorption over time. Deviations in the original absorption profile with time, suggest the sample undergoes transformation in some way, i.e. through degradation, precipitation etc. Initially, sequential UV-visible absorption measurements were conducted at room temperature for 2.6-o and 2.6-c in aqueous buffer (MOPS, pH 7.5) see Figure 2.35 (a) and (b) for a summary of the results obtained from the study. Over a two hour period, the absorption spectrum was monitored every 10 minutes. Both the ring-open 2.6-o and ring-closed 2.6-c exhibit changes in their absorbance over time. An increase in the absorbance was observed in the visible region, where 2.6-o and 2.6-c do not typically absorb, between ~650-800 nm, suggesting an increase in scattering as the sample begins to aggregate into larger particles. The bands in the UV region decreased over time; for 2.6-o a reduction in the band at 250-350 nm was observed and for 2.6-c a reduction from 300-450 nm was observed. The combination of these findings suggest that the compound is slowly aggregating and precipitating out of solution.
Figure 2.35 – Overlapped time dependent UV-visible absorption spectra collected every 10 minutes for a total of 2 hours. Showing the changes that occur in compound 2.6-o in aqueous MOPS buffer in the UV-visible absorption spectrum over a two hour time period in MOPS buffer (1% DMSO) at room temperature (a) 2.6-o (40 µM) (b) 2.6-c (40 µM). Stock solutions were prepared in 100% DMSO and then diluted prior in MOPS buffer, pH 7.5 prior to absorption measurements.

To estimate approximately how much of the compound exists in the aggregate form versus in solution, a solution of 2.6-o was prepared in MOPS buffer, a portion centrifuged and the supernatant collected for analysis using UV-visible spectroscopy. A significant deviation in the absorbance spectrum of the centrifuged solution compared to the non-centrifuged solution suggests loss of material through precipitation. Aggregate formation is concentration dependent, and beyond a particular concentration range, termed the critical aggregation concentration or CAC, molecules will predominantly exist as aggregates species. Below this concentration however, the molecules will exist primarily as monomers. The solution of 2.6-o was kept relatively dilute, 5 µM with 0.5%
DMSO. Aliquots of the 5 µM solution of 2.6-o in HEPES assay buffer were placed into centrifuge tubes. One series of tubes was centrifuged and the other was not. The commercially available BIM derivative, GF 109203X, was also analyzed for comparison, at 5 µM, 0.5 % DMSO. A speed of 15,000 rpm for 15 min was used to separate the monomer and aggregate phases. After the centrifuge process, a small but visible orange coloured pellet was visible at the bottom of the tube for 2.6-o. The supernatant was collected into a cuvette and the absorbance measured. The spectrum obtained was compared to the non-centrifuged sample of the same concentration. The resulting spectra were overlapped, see Figure 2.36, (a) and (b).

![Figure 2.36](image)

**Figure 2.36** – Changes in absorption spectrum in the presence and absence of centrifugation (15,000 rpm for 15 min). (a) 2.6-o. (b) Commercially available BIM PKC inhibitor GF 109203X. Note both compounds were diluted to 5 µM in assay buffer MOPS, pH 7.5, and contained 0.5% DMSO.

The absorption of compound 2.6-o after centrifugation is much lower in intensity than the non-centrifuged sample. The absorbance at 465 nm is 0.024 (ε = 4800 cm⁻¹M⁻¹) in the absence of the centrifugation step. In contrast, the centrifuged sample is 0.004 at 465 nm, suggesting ~6-fold decrease in concentration. The majority of the sample is removed with centrifugation. The commercially available BIM inhibitor, GF 109203X, at the same concentration gave the opposite results, there is no detectable differences in the before and after centrifuge absorption spectrum. This suggests that GF 109203X, at a concentration of 5 µM, does not form large aggregates species in aqueous solution. Though it is possible that the compound does aggregate, and the particles were not
pelleted during the centrifuge step. However, overall the data suggests that the phenylthiophene indolylmaleimide is an aggregate forming molecule.

Aggregates can often be dissolved by addition of a small amount of non-ionic surfactant to the buffer, for example Triton-X100 or Brij-35. The stability of 2.6-o was compared in HEPES buffer in the presence and absence of 0.05% Brij-35, the results are summarized in Figure 2.37. Overlap of the absorbance spectrum of 2.6-o prepared in buffer with added detergent, revealed no significant changes in the spectrum over a 2 hr time period at room temperature. There is no absorbance in the 600-800 nm region, which is expected for compound 2.6-o, and suggests the detergent prevents the formation of aggregates. By contrast, the solution that was prepared without added detergent, shows very different behaviour, and the broad absorption band extending from 400-800 nm increases over the 2 hr period, suggesting aggregate formation and precipitation.
Figure 2.37 – (a) Normalized absorbance spectrum of 2.6-o in 25 mM HEPES (pH 7.2) 1% DMSO - orange trace at t = 0 min (solid line) and t = 120 min (dotted line), and 2.6-o in 25 mM HEPES (pH 7.2) 1% DMSO + 0.05% Brij-35 – pink trace at t = 0 min (solid line) and t = 120 min (dotted line). (b) Images of solutions of 2.6-o with (+) and without (-) Brij-35 at t = 0 hours (left) and t = 6 hours (right). (c) Changes in absorbance over time at 700 nm in the presence of Brij-35 (pink diamond) and absence of Brij-35 (orange circle). (d) Changes in absorbance over time containing Brij-35 (pink diamonds, measured at 460 nm) and absence (orange circles, measured at 470 nm). The concentration of 2.6-o was 100 µM.

Images of 100 µM solutions of 2.6-o prepared in HEPES (with and without addition of Brij-35), taken immediately after preparation of the solution, and then again 6 hrs later, demonstrates the dramatic change in the solution over time as precipitate forms. There are no visible changes obvious within the 6 hr time period in the samples with detergent added, both appear as clear solutions. However, the solution without detergent appear clear initially and then a visible orange pellet is present at the bottom of the sample tube after the 6 hr period.

2.4.5.5 Dynamic light scattering

Dynamic light scattering is a technique that measures the fluctuations in light scattering intensity by particles dispersed in solution. The technique is useful for prediction of the particle’s hydrodynamic radius and can be applied as a method to help investigate if inhibitor candidates self-assemble into aggregates. Small particles in solution are subject to Brownian motion, and the distance the particles travel of a given time period combined with the total intensity of light scattered at a given angle is representative of the particle size.111

Samples of 2.6-o, 2.2-o and GF 109203X were prepared in 100% DMSO and then diluted to a concentration of 25 µM (1% DMSO) in aqueous HEPES buffer. The size distribution of the particles was measured shortly after dilution and the results obtained are summarized in the figure below.
Figure 2.38 – Size histograms (diameter) from DLS studies of the indolylmaleimides in aqueous buffer, HEPES, pH 7.5. (a) Comparison of size distribution measured for 2.2-o and 2.6-o (b) Size distribution for 2.6-o including error bars from the triplicate measurements (c) Size distribution for 2.2-o including error bars from the triplicate measurements. Samples concentration was 25 µM, HEPES, pH 7.5 (0.5% DMSO) and analysis measurements were submitted 30 min after their preparation.

The average diameter of the aggregate particles estimated from the DLS measurements for compound 2.6-o (25 µM, HEPES solution), was 284 nm. Similarly, a 50 µM solution was also prepared and the particle size estimated to be 260 nm. This suggests that beyond the critical aggregate concentration, a consistent aggregate size tends to form. Measurements of aqueous solutions that were allowed to incubate for ~1
hour gave larger sized particles >5 µm. The solution was unstable and not suitable for DLS measurements (results not included in the figure above).

Interestingly, the BIM inhibitor 2.2-o also demonstrated aggregate formation under the same conditions (25 µM, 0.5% DMSO in HEPES buffer, pH 7.5). The average diameter of the aggregate particles estimated from the DLS measurements was 170 nm. The standard inhibitor GF 109203X however at 25 µM did not appear to form aggregates in solution. Samples that aggregate in solution to form submicron sized particles typically give intense, well defined autocorrelation functions that decay on the 100-10000 µs timescale. Instead the autocorrelation function was weak and poorly defined.

2.4.6 Conclusions from this section:

The phenylthiophene indolylmaleimide derivatives examined in this section have moderate to excellent photostationary states depending on their substituents. This is an improvement compared to the unmodified BIM core, which has a very poor photostationary state. All indolylmaleimides described, show photochemical properties that are highly solvent dependent. In polar solvents, including DMSO, water, buffers etc. the photochemical ring-closure process is almost completely shut down.

The phenylthiophene indolylmaleimides tested in biochemical assays for activity against PKC β-II, displayed aggregate induced inhibition. The UV-visible absorption studies and DLS measurements suggest that over time the phenylthiophene indolylmaleimides form aggregate particles and eventually precipitate out of solution. The measured activity from the biochemical studies gave results that fluctuated depending on the time the inhibitor was stored in aqueous solutions. Addition of a non-ionic surfactant to assay buffer was found to prevent aggregate formation. Biochemical assays conducted with 0.1% Brij-35 did not show significant inhibitory effects on PKC. The phenyl thiophene may be too large or bulky to allow for efficient binding within the active site. There is additional inhibitor flexibility and potentially entropic penalty associated with addition of the phenyl group to the thiophene.
2.5 Photoresponsive inhibitor type 1B - Alkylthiophenes

The type 1A phenylthiophene series discussed in the last section, proved to be problematic for a number of reasons; not only did they exhibit poor activity towards the target enzyme, they also had limited stability in aqueous media which made assaying their inhibitory effects in vitro challenging and prone to false positives. Therefore we concluded that introduction of a phenyl thiophene group to replace one of the indole groups of the native BIM core is detrimental to binding affinity and stability in aqueous media. Encouraged by their improved photochemical performance relative to the BIM scaffold, and the efficient cycloreversion kinetics, we therefore decided to modify our design approach slightly. Keeping the thiophene substituent to conserve the photochemical properties, but to replace the phenyl substituent with a substituent that is both smaller in size and would enhance the water solubility.

2.5.1 Design and binding mode

When considering which changes may be appropriate, we predicted that a smaller sized alkylthiophene group may provide a better inhibitor candidate. Therefore in this second series, the inhibitor scaffold again consisted of the indolylmaleimide scaffold. Although the phenylthiophene was replaced with an alkylthiophene instead. The synthesis, photochemical performance, aqueous stability and enzyme inhibition behaviour is outlined in this section.

Initially, to investigate the photochemical performance of the alkylthiophene system a simple methyl substituted model compound was prepared as outlined below to determine how the photochemical properties are influenced changing from an aryl substituent to an alkyl substituent. Traditionally, aryl substituted thiophene photoswitches exhibit higher photostationary states and stability in comparison to alkylated derivatives. We therefore decided to prepare the simple methyl substituted thiophene, 2.9, as a test precursor to evaluate whether or not photochemical performance could be within a useful range for our photoresponsive inhibitor system.

Later a second alkylated thiophene was prepared in attempts to overcome some of the stability problems and lack of inhibitory activity that was encountered in the last
section. Based on the SAR data obtained from several BIM activity studies, a basic aminoalkyl chain was incorporated into the photoresponsive inhibitor structure **2.10**. The alkylamine derivative may have the added benefit of forming electrostatic interaction with the amino acid groups around the ribose binding pocket, in a similar way that has been shown for other aminoalkylated BIM derivatives co-crystallized in the active site of PKC.\textsuperscript{72-75} The presence of this group may also help to increase water solubility, which was a limitation with the previous phenyl thiophene series.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{image.png}
\caption{Target compounds methylthiophene (left) and N,N-dimethylaminopropyl thiophene}
\end{figure}

In order for the N,N-dimethylaminopropyl group of **2.10-o** to make similar interactions in the ATP binding site as alkylated bisindolylmaleimides and take advantage of the additional binding interaction with the ribose binding pocket, the thiophene group of compound **2.10-o** would have to adopt a flipped conformation. Compound **2.10-o** was aligned with the bisindolylmaleimide ligand, 2-methylBIM (co-crystallized with PKC β-II, PDB ID: 2I0E)\textsuperscript{47} to illustrate the alkyl group extending out of the pocket towards the ribose binding region.\textsuperscript{47}
Figure 2.40 – The flipped binding conformation could allow for an additional contact to be made between the inhibitor and the ribose binding pocket of the kinase (Image prepared using Pymol\textsuperscript{92} and PKC βII from PDB ID: 2I0E)\textsuperscript{47}.

2.5.2 Synthesis

Using the same synthetic route outlined for the preparation of the phenylthiophene derivatives in Section 2.4, the methyl thiophene derivative 2.40 can be easily obtained in two steps. Starting from commercially available 2,5-dimethylthiophene 2.38, a bromine atom was introduced into the 3 position by treatment with Br\textsubscript{2} and propylene oxide.\textsuperscript{112} Then in a one pot procedure, the 3-bromo-2,5-dimethylthiophene 2.39 is treated with n-BuLi, quenched with tributyl borate and then directly used in the Pd (0) catalyzed cross coupling between the thiienyl boronic ester and the N-methyl indolylmaleimide 2.40 in good yield.

Scheme 2.14 – Synthesis of methyl thiophene indolylmaleimide derivative 2.40.\textsuperscript{113} Note: conversion of 2.40 to 2.41 and 2.9 were not performed.

2.5.2.1 Preparation of N,N-dimethylamino substituted thiienyl indolylmaleimide derivative 2.10

We envisioned that 2.10 could be prepared in a similar manner as the phenylthiophene derivatives 2.4-2.8, using a Pd (0) coupling reaction to connect the thiophene bromide to the indolylmaleimide, as illustrated in Scheme 2.15.
Scheme 2.15 – Retrosynthetic analysis for the alkylamino derivative 2.10.

A potential strategy to synthesize the thiophene bromide piece 2.51, is included in the retrosynthetic analysis in Scheme 2.16. The n-propylalcohol precursor 2.49, was prepared from the α,β-unsaturated acid 2.48 using a Knoevenagel condensation between the bromomethyl aldehyde 2.47 and malonic acid.113 Followed by reduction of the double bond and acid group, to give the saturated propyl alcohol. A tosyl group was introduced to give 2.50. The dimethylamino derivative 2.51 was prepared by heating 2.50 in dimethylamine solution.

Scheme 2.16 – Retrosynthetic analysis for the bromo alkylaminothiophene piece 2.51.

In the synthesis of the previous derivatives, the N-methyl maleimide, 2.29, was used as a starting material in the cross coupling reaction with the bromothiophene. Though, this route yielded the desired product, it requires two additional steps to convert the N-methyl maleimide to the desired N-H maleimide. The hydrolysis of the N-methyl maleimide is accomplished by heating in aqueous KOH, ethanol to yield the anhydride. Followed by reaction of the product anhydride with ammonium acetate in acetic acid, to
afford the N-H maleimide final product. The steps taken to convert the N-methyl maleimide to the N-H maleimide are relatively simple to perform and high yielding, however if they could be avoided altogether, this would be more desirable. The N-methyl maleimide derivative, 2.27, is convenient during synthesis of the indolylmaleimide fragment. Compound 2.27 is soluble in the typical solvents used during construction of the BIM core (toluene) and construction of the mono-substituted indolylmaleimide (THF), whereas the N-H maleimide, 2.43, is less soluble. Additionally, it functions as a protecting group during later stages if substitution on the indole nitrogen is desired, which typically involves deprotonation of the indole nitrogen with strong base and then S_N2 reaction with a desired electrophile. Although, in this particular target molecule 2.10, the indole nitrogen is left unsubstituted, and therefore the N-methylated maleimide serves no other useful purpose during the synthesis. Therefore the cross-coupling reaction was performed using 3,4-dibromo-1H-pyrrole-2,5-dione 2.44 instead of 3,4-dibromo-1-methylpyrrole-2,5-dione 2.29, to avoid the last two steps typically required to create the N-H maleimide. We proposed that the same indolylmaleimide coupling could be conducted on the dibromo N-H maleimide in the same manner as the N-CH_3 maleimide. See below for the synthetic scheme.

Scheme 2.17 – Simplified route to synthesis of indolyl N-H maleimide piece 2.44.

The bromo maleimide is commercially available, however can be prepared simply by heating a mixture of maleic anhydride with bromine, and a catalytic amount of aluminum chloride in a sealed tube. The reaction is nearly quantitative and yields long
needles of dibromomaleic anhydride that required no further purification. The anhydride is then hydrolyzed in ammonium acetate and acetic acid, to give the N-H maleimide in moderate yield. The N-H bromo maleimide was less soluble in the desired reaction solvent THF at the same concentration as the N-CH₃ maleimide, however this could be easily overcome by diluting the reaction mixture and gently heating the THF prior to addition in the reaction sequence. The addition reaction proceeded normally and yielded ~60% (average of 5 trials) of pure product, comparable to the yields isolated with the N-CH₃ maleimide starting material.

Next, the aminoalkylated thiophene fragment was prepared. Starting from commercially available 2-methylthiophene, the aldehyde group was introduced using n-BuLi at -60°C to deprotonate α-proton of thiophene, followed by quenching of the resulting species using DMF and hydrolysis with dilute HCl. This gave the 5-methylthiophene-2-carbaldehyde 2.46, in good yield. A bromine atom was introduced using liquid Br₂ in acetic acid. The product 2.47, was then refluxed with malonic acid in pyridine and piperidine to give the corresponding α,β-unsaturated acid 2.48 in good yield, using a Knoevenagel condensation. The α,β-unsaturated acid was then reduced using lithium aluminum hydride to give the alcohol 2.49. The alcohol was activated by introducing the tosylate group, which would serve as a good leaving group in the next step. Lastly, reaction of 2.50 with dimethylamine solution in THF gave the final product, the thiophene fragment 2.51.
Scheme 2.18 – Synthesis of alkylaminothiophene fragment 2.51.

In the final coupling step, the alkylamino fragment 2.51 was treated with \( n\)-BuLi, quenched with tributylborate and the corresponding boronic ester intermediate was used directly in the Suzuki cross coupling step, along with the bromoindolylmaleimide 2.44, potassium carbonate and \( \text{Pd(PPh}_3\text{)}_4 \). Refluxing for several hours yielded the desired product 2.10 in satisfactory yield, 38% as a vibrant orange powder.

Scheme 2.19 – Synthesis of 2.10 from a Pd(0) catalyzed cross-coupling reaction between the boronic ester of alkylaminothiophene 2.51 and 3,4-dibromo-1H-pyrrole-2,5-dione 2.44.
2.5.3 Photochemical Properties

2.5.3.1 UV-visible absorption properties of series 1-B inhibitor scaffold

Knowing that indolylmaleimides typically exhibit solvent dependent photostationary states, the photoinduced switching behaviour of the methyl derivative 2.40 was first investigated in hexanes solution, since this solvent’s relatively low dielectric constant tends to permit higher photostationary states in comparison to more polar solvents. A 50 µM solution of 2.40 in hexanes/THF (9:1) was prepared, and its absorption changes with light irradiation are displayed in Figure 2.41. The ring-open isomer has an absorption band in the visible region, at 441 nm, (6025 cm⁻¹ M⁻¹) within the typical range expected for bisarylmaleimides. With light exposure, the peak at 441 nm decreases in intensity slightly, and this change is accompanied by new peaks at 530 nm and 386 nm from the newly formed 2.40-c. The ring-closure process was also investigated in THF and DMSO, the results displayed in Figure 2.41 (b) and (c) respectively. The light induced changes that occurred in the spectrum when dissolved in THF resembled the hexanes solution described above. The absorption band of the ring-open isomer appeared slightly red shifted at 443 nm, and the ring-closed isomer bands appeared at 545 nm and 390 nm. When performing the ring-closing reaction in DMSO, the absorbance spectrum did not change very much, suggesting that the solution consists predominantly of ring-open isomer. This observation was expected based on the data obtained from the phenylthiophene derivatives in the previous series.
Figure 2.41 – (a) Changes to the UV-visible absorption spectrum of compound 2.40-o, 50 µM when irradiated with 450 nm light at 60 second intervals and dissolved in (a) hexanes/THF (9:1), (b) THF, (c) DMSO (d) Overlap of the changes in absorbance at $\lambda_{\text{max}}$ of the ring-closed isomer as a solution is irradiated with 450 nm light ($\lambda_{\text{max}}$ hexanes = 540 nm, $\lambda_{\text{max}}$ THF = 550 nm, $\lambda_{\text{max}}$ THF = 580 nm).

Similarly, the UV-visible absorption measurements were conducted with the N,N'-dimethylaminopropyl substituted thiophene derivative, 2.10. The absorption spectra of 2.10 were recorded in hexanes, THF and DMSO, see Figure 2.42 (a-c) for comparison. Overall, the absorption spectrum resembles the methyl derivative 2.40. In DMSO, the changes that occur with light irradiation are expectedly very small indicating a poor photoconversion in this polar solvent. The ring-closure kinetics are plotted as a function of the $\lambda_{\text{max}}$ of the ring-closed isomer versus time. There is a small difference in the
maximum absorbance achieved in hexanes versus THF, and a very substantial difference between these and DMSO.

![Chemical structure](image)

**Figure 2.4** – a) Changes to the UV-visible absorption spectrum of compound 2.10-o, 50 µM when irradiated with 450 nm light at 60 second intervals and dissolved in (a) hexanes/THF (9:1), (b) THF, (c) DMSO (d) Overlap of the changes in absorbance at $\lambda_{\text{max}}$ of the ring-closed isomer as a solution is irradiated with 450 nm light ($\lambda_{\text{max}}$ hexanes = 540 nm, $\lambda_{\text{max}}$ THF = 525 nm, $\lambda_{\text{max}}$ THF = 580 nm). (d) changes in absorption over time for THF (A = 540), for hexanes/THF (9:1) (A = 525 nm), and DMSO (A = 575 nm).
Table 2.7 – Summary of UV-visible absorption data for compounds 2.10 and 2.40

<table>
<thead>
<tr>
<th>solvent</th>
<th>2.10-o</th>
<th>2.10-c</th>
<th>2.40-o</th>
<th>2.40-c</th>
</tr>
</thead>
<tbody>
<tr>
<td>hexanes/THF (9:1)</td>
<td>436 (5000)</td>
<td>525, 388</td>
<td>443 (6000)</td>
<td>535, 388</td>
</tr>
<tr>
<td>THF</td>
<td>440 (4800)</td>
<td>540, 392</td>
<td>448 (6000)</td>
<td>540, 392</td>
</tr>
<tr>
<td>DMSO</td>
<td>448 (5400)</td>
<td>~575</td>
<td>455 (5400)</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

2.5.3.2 Fluorescence emission spectroscopy

The fluorescence emission spectrum of compound 2.10-o, like the phenylthiophene series, is dependent on solvent polarity. The change in the emission wavelength when changing the solvent polarity was measured for compound 2.10-o, see Figure 2.43 overlapping the emission peaks in hexanes, THF and DMSO. In hexanes an intense emission band appears at 535 nm, whereas in THF the emission wavelength is shifted to 565 nm, and in DMSO the band appears at ~600 nm, significantly broadened and intensity diminished substantially.

Figure 2.43 – Comparison of the changes in the emission wavelength of 2.10-o in solvents of different polarities. Compound 2.10-o, 10 µM in THF/hexanes (9:1), 100% THF, and DMSO.
2.5.4 Stability in aqueous media

2.5.4.1 Aqueous stability

The last chapter outlined the importance of investigating the stability of the inhibitor in aqueous media, particularly to identify whether or not the compound may aggregate in solution during the *in vitro* enzyme studies and make measurement of their influence on enzyme activity challenging. The stability of the compounds in aqueous media were tested prior to assay screening to check for signs of aggregate formation. A summary of the time dependent UV-visible absorption data for compound 2.10-o and 2.10-c in HEPES buffer (pH 7.5, 1% DMSO) monitored over a 2 hours time period are given in Figure 2.44. The photochemical preparation and isolation of compound 2.10-c are described in the next section. For both 2.10-o and 2.10-c, the UV-visible plots show very little change in absorbance over time, as can be seen from the nearly overlapping absorbance plots throughout the 2 hr duration of the experiment. There is however a small decrease in the absorbance 535 nm for 2.10-c, and 450 nm 2.10-o see zoom in inset traces in Figure 2.44 (a-b). There could indicate that some precipitation is occurring over time, however for the most part, the spectrum remains constant. The results are a substantial improvement compared to 2.6 in the phenylthiophene series.

The lack of changes in 2.10-c over time also indicate that the ring-closed isomer does not spontaneously revert to the ring-open 2.10-o isomer in the dark. The thermal stability of 2.10-c is critical for *in vitro* enzyme studies later, so that the compound can be kept in the inactive state until desired activation with light.
2.5.4.2 Dynamic Light Scattering Measurements

Dynamic light scattering (DLS) was also used to evaluate the stability behaviour of the synthesized inhibitor 2.10 in solution. As was described earlier, DLS is useful for detection of nanometer to micron sized particles and can be useful for investigating whether 2.10 self-assemble into aggregates in the assay buffer system used during
kinase activity measurements. The results from the UV-visible studies suggest that 2.10 is soluble in aqueous media, however DLS measurements were also included to support this observation. Two solutions of 2.10-o, at concentrations of 25 µM (0.5% DMSO) and 50 µM (1% DMSO) in HEPES assay buffer were prepared and allowed to stand for 30 minutes prior to analysis. The DLS output from the 50 µM 2.10-o measurement is displayed in Figure 2.45 below.

![Figure 2.45](image)

**Figure 2.45** – Autocorrelation function measured from DLS studies of 2.10-o in HEPES buffer, pH 7.5 at 50 µM (1% DMSO).

At both of the concentrations tested for 2.10-o, the presence of aggregate particles in solution were not detected. Like the data obtained from inhibitor GF 109203X, the autocorrelation function was poorly defined and the measured signal intensity was very low. A low signal intensity for the sample suggests that the medium scatters light very weakly, which is expected if 2.10-c is solubilised in the HEPES buffer to give a homogeneous solution. The decay of the autocorrelation function is used to predict the size of the particle. A low concentration of scattering species in the solvent medium will also give a poor autocorrelation function since there is not an adequate amount of particles present in solution to detect.
2.5.5 Ring-closed isomer isolation

2.5.5.1 Synthesis

An ideal inhibitor should possess a high conversion yield between the two possible isomeric states so that complete “on” and “off” photoswitching can be achieved. However, nearly quantitative photoconversion is usually not possible in both states. This is true for the compounds featured in this series, where only the ring-open isomer can be produced in high yield. The ratio of ring-closed isomer can only be enriched with light. In cases where the inactive or “off” state is the more thermodynamically stable isomer, and exists predominantly under ambient lighting conditions, a high photostationary state may not be required. Some percentage of active isomer can be generated with light exposure, which may be sufficient to exert the desired effect. The remainder of inactive isomer does not interfere with the application. This type of “on” and “off” conversion is not as efficient, and larger quantities may have to be used to achieve the desired effect. For the inhibitors featured in this chapter, the ring-open and more thermodynamically stable isomer is the active state. Therefore to assess “on” and “off” control over enzyme activity, a high conversion yield is absolutely necessary.

The results from this chapter indicate that the photostationary states range from excellent to moderate, when using non polar organic solvents (i.e. hexanes and THF). However, when using more polar solvents, including water and aqueous buffer, the photoconversion yield is very poor. Only aqueous media can be used for conducting the assay studies. Therefore, in order to investigate the effects of the both individual isomers, the ring-closed isomer must be isolated from the photostationary state mixture, so that the active ring-open isomer does not interfere with measurement of the “off” state.

Separation of the photostationary state mixture can be usually be achieved chromatographically. It requires that the ring-closed isomer is stable and does not revert back to the ring-open isomer simultaneously. Additionally, the two isomers must have different Rf values, so that they can be separated using techniques such as column chromatography. For isolation of pure 2.10-c, a desired quantity of the ring-open isomer 2.10-o can be converted to the photostationary state in a non-polar solvent that permits
an acceptable photoconversion yield, for example, in THF or hexanes. Followed by removal of that solvent, purification using column chromatography in the desired eluent system, removal of the eluent solvent and then finally dissolution into DMSO, for use in vitro enzyme studies.

The ring-closed isomer, 2.10-c has a higher Rf value than the ring-open isomer 2.10-o, see image taken below of a TLC plate of the photostationary state mixture (Figure 2.46). The ring-closed isomer 2.10-c appears as a reddish colour, in comparison to the ring-open which appears as an orange/yellow colour. This reddish colour change is expected based on the UV-visible absorption band that appears at 560 nm, upon exposure of a solution of the ring-open isomer to blue light (~450 nm). Column chromatography (silica gel, DCM/MeOH 2.5%/TEA 2.5%) was used to isolate the ring-closed isomer. Smaller percentages of TEA were also tested, however these did not give sufficient separation between the two isomers.

Figure 2.46 – Representative TLC of compound 2.10-o and 2.10-c, showing the differences in Rf values of the ring-closed and ring-open isomers.

The product was isolated after column chromatography yielded a deep red/purple amorphous solid. Like all of the ring-closed isomers in the indolylmaleimide series, 2.10-c is very sensitive to light. Therefore all manipulations involving 2.10-c were performed in the dark to prevent conversion to the ring-open isomer 2.10-o (a dim red light source was user to see reagents and equipment).
2.5.5.2 NMR Characterization

Comparison of the $^1$H NMR spectrum of the ring-closed and ring-open isomers revealed that there are several distinct changes in the spectrum that accompany the photoisomerization, see Table 2.8 below for a summary of the resonances for 2.10-o and 2.10-c. The aromatic region of the two spectra from 6.5-12.5 ppm are also included below in Figure 2.47. Significant differences can be observed in the indole N-H, and the indole protons at the 3 and 4 positions (labelled 3, 4 and 3', 4' in Figure 2.47). The indole N-H proton of the ring-open isomer, 2.10-o, appeared as a broad singlet at 11.50 ppm. However, in the ring-closed isomer, 2.10-c, the indole N-H is shifted upfield to 6.70 ppm, reflecting the change in electronic distribution and the loss of aromaticity with ring-closure. The maleimide N-H of the ring-closed isomer does not change substantially, there is a ~0.1 ppm shift downfield relative to the ring-open isomer. The protons on indole labelled 3 and 3’ are shifted downfield in by ~1.05 ppm with ring-closure, with 3 appearing at ~7.28 ppm in 2.10-o and at 8.33 ppm in 2.10-c. The 3’ doublet becomes more deshielded as the molecule is forced into a nearly planar conformation, and the 3’ proton is within close proximity to the maleimide carbon oxygen double bond. This pattern is typically observed in the indolo[2,3-a]carbazole framework, and helps to support predicted structure of the ring-closed.
Figure 2.47 – Overlap of partial $^1$H NMR spectra for compounds 2.10-c (top) and 2.10-o (bottom) in DMSO-d$_6$, 400 MHz.

Table 2.8 – Comparison of $^1$H NMR chemical shifts for compounds 2.10-o and 2.10-c in DMSO-d$_6$, 400 MHz

<table>
<thead>
<tr>
<th>Proton #</th>
<th>ring-open (2.10-o)</th>
<th>ring-closed (2.10-c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11.50, 1H (bs)</td>
<td>6.70, 1H (bs)</td>
</tr>
<tr>
<td>2</td>
<td>10.96, 1H (bs)</td>
<td>11.07, 1H (bs)</td>
</tr>
<tr>
<td>3</td>
<td>7.29-7.27, 1H (dd)</td>
<td>8.34-8.32, 1H (dd)</td>
</tr>
<tr>
<td>4</td>
<td>7.03-6.99, 2H (m)</td>
<td>7.25-7.21, 1H (td)</td>
</tr>
</tbody>
</table>
To characterize the photochemical behaviour and stability of the product, **2.10-c**, a dilute solution in THF was prepared for UV-visible absorption measurement, the spectrum is shown in Figure 2.48 (b). There is a broad absorption band 535 nm, a second strong, well defined peak appears at 393 nm, and third smaller sized peak at 320 nm. The ring-closed isomer 2.10-c resembles the photostationary state spectrum, except that it is more intense since there is no contaminating ring-open isomer. The solution is purple/red in colour, see Figure 2.48 (c). After exposure of 2.10-c to >490 nm light, 2.10-o is regenerated quantitatively within a few seconds. A band at 438 nm appears from the ring-open isomer 2.10-o, and the solution changes to a yellow colour.
Figure 2.48 – (a) Conversion of the pure ring-closed isomer isolated using column chromatography to the ring-open isomer with yellow light (> 490 nm cut-off filter). (b) Comparison of the UV-visible absorption spectrum of solution of 2.10-c (14 µM, THF). Irradiation with > 490 nm light regenerates 2.10-o, and subsequent exposure of 2.10-o to 450 nm light, generates the photostationary state mixture. (c) Images of the ring-closed 2.10-c and ring-open 2.10-o in THF (14 µM).

Subsequently, the solution was irradiated with 450 nm light to reach the photostationary state mixture. Using Beer Lambert’s law and the molar extinction coefficient ($\varepsilon = 5520$ L mol$^{-1}$ cm$^{-1}$ at $\lambda_{\text{max}} = 530$ nm in THF) for the pure ring-closed isomer, the photostationary state can be estimated from this spectrum. The photostationary state was calculated to be $\sim 42\%$ for conversion of 2.10-o to 2.10-c.
2.5.6 Enzyme Studies

2.5.6.1 Assay development

A FRET-based assay was used to measure the enzyme activity of compound 2.10. Some of the inhibitors from the previous phenylthiophene series were also evaluated using this assay to compare their activities to compound 2.10.

A commercially available Z'-LYTE kinase activity kit – serine/threonine 7 peptide protocol was purchased from Invitrogen (Catalogue no: PV 3180, Invitrogen Corporation, Carlsbad, CA, USA). Briefly, in the first stage of the assay, the protein kinase was incubated with a synthetic peptide substrate labelled with a coumarin donor and a fluorescein acceptor FRET pair. In the non-phosphorylated state, the peptide was cleaved in the second development stage of the assay disrupting the FRET pair, and giving rise to an increase in coumarin emission. In the phosphorylated state, the presence of the phosphate group suppresses peptide cleavage and the FRET process dominates. The phosphorylation reaction progress was calculated from the ratio of the measured coumarin emission to fluorescein emission. Kinase assays were performed in uncoated, black, flat bottom 96 well plates. PKC βII (0.5 µg/mL) was treated at room temperature for 1 h with Z'-Lyte Ser/Thr 7 peptide (2 µM), ATP (25 µM) and with either inhibitor samples at various concentrations or with DMSO control (1%). The assay buffer contained HEPES buffer (25 mM, pH 7.5), MgCl₂ (10 mM), EGTA (1 mM), 0.01% Brij-35. An lipid/Ca²⁺ mixture was used to activate the kinase prior to phosphorylation reaction. Unless otherwise stated, the activator mixture contained the following: phosphatidylserine (54 µg/mL), 1-stearoyl-2-linoleoyl-sn-glycerol (3.5 µg/mL), 1-oleoyl-2-acetyl-sn-glycerol (5.2 µg/mL) and CaCl₂ (83 µM). The activator mixture was sonicated for 60 s prior to adding it to the assay buffer. The development reagent was added to the wells and incubated at room temperature for 1 h followed by the stop solution. The degree of phosphorylation was calculated by measuring the emission ratio of coumarin (λ<sub>exc</sub> = 400 nm, λ<sub>em</sub> = 445 nm) and fluorescein (λ<sub>exc</sub> = 400 nm, λ<sub>em</sub> = 520 nm) for each well. Emission ratios were measured on a BioTek Synergy™4 Hybrid Microplate Reader.

A figure illustrating the assay design is given in Figure 2.49 a-b. The FRET peptide gives a weak emission peak at 520 nm when excited with 400 nm light due to
FRET, see the green trace in the fluorescence emission spectrum (Figure 2.49, b). During step 1 of the assay, in the presence of active kinase (and ATP), substrate is phosphorylated. Later on in step 2, a protease is added to cleave a specific sequence on the FRET peptide. The presence of a phosphate group on the peptide hinders access to the cleavage site, and the FRET process dominates. However, any unreacted starting material (as is expected when the protein kinase reaction is conducted in the presence of a good inhibitor), the non-phosphorylated peptide is cleaved by the protease, separating the FRET pair. As a result, excitation with 400 nm gives a large increase in emission intensity at ~445 nm from the coumarin fluorophore.

![Diagram](image)

**STEP 1:** kinase assay
- Substrate $\rightarrow$ Phosphorylated substrate
- Protein kinase + ATP

**STEP 2:** development reaction
- Phosphorylated substrate $\rightarrow$ Non-phosphorylated substrate
- Protease
- $\lambda_{ex} = 400$ nm
- $\lambda_{em} = 450$ nm / 525 nm

**MEASUREMENT:**
- $\lambda_{ex} = 400$ nm
- $\lambda_{em} = 450$ nm / 525 nm

**EMISSION:**
- Phosphorylated substrate + protease
- Non-phosphorylated substrate + protease
Figure 2.49 – (a) Cartoon illustrating the assay concept. A protein kinase peptide modified with a coumarin donor and a fluorescein acceptor, gives weak emission due to FRET. When the peptide is phosphorylated during the kinase reaction (step 1), the phosphate group slows protease cleavage of the peptide in the development reaction (step 2). (b) Fluorescence emission of FRET-peptide, non-phosphorylated (green trace) showing quenching of coumarin emission by fluorescein residue on the opposite end of peptide and after addition of the development reagent (blue trace), where coumarin emission predominates due to the absence of FRET.

The ratio of the emission at 450/525 nm with 400 nm excitation is a direct measure of the kinase activity. Using the following controls: 100% phosphorylated peptide and 0% phosphorylated peptide starting material, a calibration can be made to determine the percentage of peptide phosphorylated under the assay conditions. See equations below for calculation used:

Equation 2.1
\[
\text{Emission ratio} = \frac{\text{coumarin emission (445 nm)}}{\text{fluorescein emission (520 nm)}}
\]

Equation 2.2
\[
\% P = \frac{(\text{emission ratio} \times F_{100\%}) - C_{100\%}}{(C_{0\%} - C_{100\%}) + [\text{emission ratio} \times (F_{100\%} - F_{0\%})]}
\]

where:

% P = % phosphorylation

\(C_{100\%}\) = average coumarin emission signal of the 100% phosphorylation peptide control

\(C_{0\%}\) = average coumarin emission signal of the 0% phosphorylation peptide control

\(F_{100\%}\) = average fluorescein emission signal of the 100% phosphorylated peptide control

\(F_{0\%}\) = average fluorescein emission signal of the 0% phosphorylation peptide control
Prior to testing the inhibitory activity of the synthesized compounds against PKC, optimization of the assay parameters was conducted first to determine suitable concentrations of substrate, ATP, and lipid activators. The introduction to this chapter outlined the importance of the combination of phospholipid cofactors and calcium required for activating PKC from an inactive condensed state to a catalytically competent kinase. In the absence of appropriate concentrations of cofactors, the kinase will remain in the inactive state, where the ATP site is not solvent accessible.

As was outlined earlier in the chapter, PKC must be in its active open conformation for substrate phosphorylation to take place. In the cell, PKC is recruited from the cytosol to the membrane in the presence of increased Ca$^{2+}$ levels. Upon interaction with the membrane lipids, via the DAG and phosphatidylserine binding sites in the regulatory domain (regions C1 and C2 of PKC β-II), this induces a change in conformation of the kinase, releasing/dissociating the pseudosubstrate, and opening up the enzyme structure to its catalytically active form.\textsuperscript{64} It is well documented that addition of lipid cofactors and calcium are required for activation of PKC β-II \textit{in vitro}. Presumably, the lipids form micelle-like structures in solution for the kinase regulatory domain to interact with, and generate the catalytically competent form of the enzyme.\textsuperscript{64a-c} The nature of the activation mechanism \textit{in vitro} and \textit{in vivo} are still under debate, and there are a variety of different procedures described in literature for activation of the enzyme.

There was some ambiguity as to whether or not the PKC could be stimulated once with the appropriate concentration of cofactors prior to dilution in the assay, or if the kinase activation process is transient and reversible, requiring the cofactor concentration be maintained throughout the duration of the assay. The range of cofactor concentrations reported in literature procedures for \textit{in vitro} activation of PKC for biochemical studies is very large, so it was not immediately clear what cofactor concentration was ideal for use.

Prior to screening inhibitors a series of studies were conducted varying the concentration of the activating lipid mixture. In the first study, two concentrations of ATP were tested, 25 µM and 12.5 µM and a one time activation of the kinase with a Ca$^{2+}$ and lipid mixture. The lipid composition used during the phosphorylation reaction is listed in
Table 2.9. Study 1. The lipid mixture is added to the stock kinase, given ~ 15 minutes to incubate and then further diluted into the reaction mixture. The phosphorylation reaction was initiated with ATP and allowed to run for 1 hour at room temperature. The results are summarized in the figure below.

Figure 2.50 - Protein kinase standard curve of the percentage phosphorylation versus the concentration of PKC β-II. The assay was run with a constant amount of substrate, for 1 hour at RT. The kinase was activated with a mixture of Ca^{2+} and phospholipids at one point before dilution into the reaction mixture. Data points represent a single measurement value. The solid lines are included only to aid the reader with viewing the different ATP concentrations.

The results from the standard curve show a linear increase in phosphorylation with quantities of ~20-25 ng of PKC β-II under the specified conditions. The quantity of phosphorylated product is higher with 25 µM added ATP, a trend that is expected. The assay kit recommends using a range of 20-40% phosphorylated product to screen inhibitors, therefore at both concentrations of ATP, a ~10-20 ng of kinase would be reasonable under these particular conditions.

In the second and third study, the amount of lipid activator was increased, see table below for the quantities of the lipid, calcium, mixtures tested. The stock kinase is initially diluted, and then a constant volume of lipid activating mixture was added to the assay wells just prior to initiating the assay. Kinase was given ~15 min incubation time before initiating the phosphorylation reaction with ATP addition.
Table 2.9 – Summary PKC activating mixture components used in the assays

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Study 1</th>
<th>Study 2</th>
<th>Study 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidyserine (µg/mL)</td>
<td>24.0</td>
<td>108.1</td>
<td>54.1</td>
</tr>
<tr>
<td>1-stearoyl-2-linoleoyl-sn-glycerol (µg/mL)</td>
<td>1.5</td>
<td>6.7</td>
<td>3.4</td>
</tr>
<tr>
<td>1-oleoyl-2-acetyl-sn-glycerol (µg/mL)</td>
<td>2.3</td>
<td>10.5</td>
<td>5.2</td>
</tr>
<tr>
<td>CaCl₂ (µM)</td>
<td>37.0</td>
<td>166.7</td>
<td>83.3</td>
</tr>
<tr>
<td>DTT (µM)</td>
<td>18.5</td>
<td>83.3</td>
<td>41.7</td>
</tr>
</tbody>
</table>

Note: In all cases, the lipids were vortexed for 3 x 30 sec to generate vesicles. The lipid vesicles were then dispersed with brief sonication and used in the assay.

The results from the standard curve using the conditions outlined in Table 2.9 for studies 2 and 3 were quite different from study 1 which used less of the activating lipid / Ca²⁺ mixture throughout the phosphorylation reaction. In both study 2 and 3 (Figure 2.51 (a) and (b) respectively) there is a higher percentages of phosphorylation over the one hour period. Even dilute range of concentration of PKC tested, ~0.4 ng / assay, phosphorylated ~25% of substrate. There is a linear increase in phosphorylation with increasing quantities of PKC β-II, however the curve reaches saturating conditions with ~5 ng of PKC in the presence of 25 µM of ATP and ~10 g of PKC in the presence of 12.5 µM and 6.25 µM ATP. The quantity of phosphorylation product is higher with 25 µM added ATP, this trend is expected. The assay kit recommends using a range of 20-40% phosphorylated product to screen inhibitors, under these conditions the kinase is much more active and 0.5-5 ng of PKC are appropriate for screening studies based on this data obtained in the standard curve.
Figure 2.5 – Protein kinase standard curve of the percentage phosphorylation versus
the concentration of PKC β-II. (a) Study 2, containing the highest concentration of activating cofactor mixture. (b) Study 3, containing an intermediate concentration of activating cofactor mixture. In both studies The assay was run with a constant amount of substrate, for 1 hour at RT. Three concentrations of ATP were tested, 25 µM, 12.5 µM and 6.25 µM. Data points represent a single measurement value. The solid lines are only included to aid the reader with viewing the different ATP concentrations.

Based on the results from the standard curves using different amounts of a lipid cofactor mixture, the kinase activity is highly dependent on the concentration of the activators during the entire assay. Studies that used the higher concentrations of activating lipid mixture throughout the assay were more efficient at phosphorylating substrate than the kinase using the conditions outlined in study 1 (activating mixture was added to the stock kinase, and then the kinase diluted further into the kinase assay mixture). Dilution of the lipids will no doubt alter their morphology and the composition of the micelles produced. Below a critical concentration, the lipids will not form the micellar structures necessary for PKC activation. Therefore, when screening inhibitors we opted to use the conditions outlined in study 3, which contain a moderate amount of lipid activator mixture to ensure the ratio of active kinase remains high in the assay.

2.5.6.2 Inhibitor screening

The inhibitory activity studies were performed for compounds 2.10-o and 2.10-c, as well as some of the compounds from the last section featuring the phenylthiophene series. Solutions of inhibitors were prepared in 100% DMSO, at 30X and then diluted to the desired concentrations in buffer to 3X, and then used in the assay. The
concentration of DMSO in the assay was kept at 1%. In order to reduce the effects of aggregation, the buffer was prepared with 0.01% Brij-35 detergent. A screening study was conducted on the following compounds in their ring-open states, at 1 µM (1% DMSO). The results of the study are summarized below in Table 2.10.

Table 2.10 – Summary of activity for selected compounds 2.10-o and selected compounds from the phenylthiophene series against PKC β-II

<table>
<thead>
<tr>
<th>Compound</th>
<th>% Inhibition at 1 µM (phosphorylation % relative to DMSO control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.4-o</td>
<td>1</td>
</tr>
<tr>
<td>2.5-o</td>
<td>7</td>
</tr>
<tr>
<td>2.6-o</td>
<td>1</td>
</tr>
<tr>
<td>GF 109203X</td>
<td>98</td>
</tr>
<tr>
<td>2.10-o</td>
<td>68</td>
</tr>
</tbody>
</table>

Note: The assay conditions used were as follows, each well contained 5 ng of PKC β-II (1.6 nM) was treated at room temperature for 30 minutes with Z'-Lyte Ser/Thr 7 peptide (2 µM), ATP (12.5 µM) and with either inhibitor samples at various concentrations or with DMSO control (1%). The assay buffer contained HEPES buffer (25 mM, pH 7.5), MgCl$_2$ (10 mM), EGTA (1 mM), 0.01% Brij-35 and an activator mixture with phosphatidylserine (54 µM), 1-stearoyl-2-linoleoyl-sn-glycerol (3.4 µM), 1-oleoyl-2-acetyl-sn-glycerol (5.2 µM), CaCl$_2$ (83.3 µM) and DTT (41.7 µM). The activator mixture was vortexed for 3 x 30 seconds and then sonicated for 60 s prior to adding it to the assay buffer. All inhibitor samples were given 15 minutes to incubate with the kinase prior to initiation of the phosphorylation reaction with ATP.

The results obtained from the screening were promising for the newly synthesized inhibitor 2.10-o which inhibited ~68% of the kinase activity relative to the DMSO control. The activity is not as good as the inhibitor GF 109203X with ~98% inhibition under the same conditions. However, for the goals of the chapter the reduction in activity observed with 2.10-o is a substantial improvement, compared to the phenylthiophene series which all exhibited <10% inhibition at 1 µM.

2.5.6.3 Light controlled activation of inhibitory activity

The inhibitory activity of compound 2.10-o was investigated further. Its concentration was varied from 25 µM to 0.05 µM and then assayed in duplicate using the
same conditions described for the screening study in the last section 2.5.6.2. The dose-response curve obtained during the assay is shown below in Figure 2.52 (a). The data was fitted to a dose-response curve (using the variable slope, four parameter function in Prism, version 7.0, GraphPad Software Inc, 1995-2016). The estimated IC\textsubscript{50} was 0.58 µM for compound 2.10-o and a Hill slope of 1.1. The inhibition does not appear to completely reach zero at the higher range of concentration tested.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2_52}
\caption{(a) Log of the inhibitor concentration 2.10-o (µM) versus the percentage relative activity of the control sample, (1% DMSO). (b) Log of the inhibitor concentration (µM) versus the percentage relative activity of the control sample, (1% DMSO). Compounds 2.10-c (pink trace) and 2.10-c + exposure to yellow light (>490 nm cut-off filter) (purple trace). Error bars represent the mean ± standard deviation of duplicate samples.}
\end{figure}

A second assay was conducted to include the ring-closed isomer 2.10-c and 2.10-c exposed to yellow light (>490 nm filter) for 30 seconds prior to measurement in the assay. In both cases, several concentrations of ring-closed were prepared, ranging from 25-0.1 µM for 2.10-c and ranging from 25-0.2 µM for 2.10-c exposed to yellow light. Due to the light sensitivity of 2.10-c, the entire assay was performed in the absence of the ambient lab lighting which emits light sufficient to induce cycloreversion with a few minutes. Instead, a red coloured light was used to see the equipment and assay reagents (light was placed ~1 m away from assay reagents).

The dose-response curve obtained for 2.10-c is shown in Figure 2.52 (b) along with light exposed sample of 2.10-c. The results that 2.10-c is less effective at inhibiting PKC β-II, the activity is not completely abolished, as there is a small decrease in activity ~10-25%, relative to the control sample (containing 1% DMSO) suggesting that 2.10-c
may inhibit the phosphorylation reaction to some extent. The light triggered activation of 2.10-o from 2.10-c is also displayed in Figure 2.55 (b). The dose-response curve obtained from the light irradiated sample resembles that obtained from 2.10-o shown in Figure 2.55 (a). This indicates that light triggered conversion of the less active inhibitor 2.10-c to the more active inhibitor 2.10-o can be achieved within 30 seconds exposure to visible light, effectively demonstrating our concept.

2.5.7 Conclusions from this section:

This section of the chapter focused on improving the inhibitory activity towards PKC and the aqueous stability of the inhibitor targets. The phenyl substituents that were used in type-1A were replaced with an alkyl group. The N,N-dimethylaminopropyl group of compound 2.10 proved to be effective for enhancing the aqueous buffer stability, as the time dependent UV-visible absorption traces did not show changes in peak intensities (at least over a 2 hour time period, long-term stability was not confirmed). Solutions of 2.10 in both the ring-open and ring-closed forms did not appear to aggregate and precipitate over time either.

However, as expected, the photochemical behaviour of the inhibitor targets were relatively similar to the phenylthiophene series prepared earlier. The alkylthiophene indolylmaleimide derivative 2.9 and 2.10 have moderate photostationary states ~40%. The photochemical properties are also highly solvent dependent, in polar solvents including DMSO, water, buffers etc, photoswitching is hindered. This required that photostationary state be achieved in a non-polar solvent, the two isomers separate by column chromatography and then re-dissolved in DMSO before use in biochemical studies.

The biochemical assays for activity against PKC β-II revealed that 2.10-o had exhibited fairly good inhibitory activity, with a measured IC$_{50}$ of 0.58 µM. In comparison to earlier derivatives, the removal of the phenyl group and replacement with an alkyl group improved the activity substantially. However, compared to the traditional bisindolylmaleimides, the presence of the thiophene group appears to reduce activity in comparison to the traditional bisindolylmaleimide scaffold. Although the inhibition
mechanism of $2.10-o$ was not confirmed, if ATP competitive, the size, shape, rotational flexibility and electronic properties of thiophene are expectedly quite different from indole. The thiophene may form less favourable interactions with the binding pocket and as a consequence, lower activity is observed.

However, despite the comparatively lower activity of $2.10-o$ compared to the traditional bisindolylmaleimides, the inhibitory activity was still modifiable with light energy, which is the main goal of the project. The ring-closed isomer $2.10-c$ was isolated from the photostationary state mixture by column chromatography and assayed again PKC. The inhibitory performance of $2.10-c$ was poor, as expected, and although it decreased phosphorylation activity to some degree, the highest concentration tested 25 µM displayed ~10% inhibition. In contrast, the ring-open isomer inhibited activity ~83% at this concentration. The light activation of inhibition was also conducted for $2.10-c$. Solutions of $2.10-c$ were exposed to yellow light (generated with a > 490 nm cut-off filter), to induce cycloreversion, prior to measurements in the kinase assay. The dose-response data obtained from these measurements show a similar trend similar to the ring-open isomer $2.10-o$. We demonstrate that it is possible to convert the photoswitchable derivative from a less active state, $2.10-c$ to a more active state $2.10-o$ using brief exposure to low intensity yellow light.

### 2.6 Type 2 – Bisindoles

The last section demonstrated that the replacement of one indole group with a thiophene group, generated a compound with moderate to excellent photocyclization yields in non-polar solvents only. Considering that the cyclization yields are negligible in polar solvents, for instance DMSO and water, this feature eliminates the possibility for complete “on” and “off” photoswitching in real time measurements. A derivative that would allow for reversible conversion from the ring-closed and the ring-open state in both polar and non-polar solvents was next investigated.

The electronic nature of the indolylmaleimide scaffold contributes to the poor photochemical performance. Another approach to influence the photochemistry of the inhibitor compound is to adjust the electronic structure of the BIM core. This can be done
through modification to the central maleimide ring. The maleimide group hydrogen bonds with 2-3 amino acids within the active site of the kinase. Based on SARs reported for BIMS, typically substitution or modification of the maleimide is not well tolerated.\textsuperscript{68} However, we considered that a lactam structure could be a possible candidate for the photoresponsive inhibitor series since it would allow an opportunity to enhance photochemical performance in aqueous media.

2.6.1 Introduction and design

In this inhibitor series, our goal was to investigate how modification of the maleimide would influence the photocyclization behaviour, and in particular, determine if reducing one of the maleimide C=O to either a lactam or a hydroxylactam would change the electronic nature of the molecule and generate an inhibitor that permits photoswitching in polar solvents, for instance water, DMSO etc. This would potentially allow for the opportunity for the user to switch the activity of the inhibitor to be reversibly toggled between an inactive “off” state to an active “on” state. This reversibility feature would increase the range of applications for this technology.

Figure 2.53 – Modification of the maleimide C=O group to yield the corresponding lactam, 3,4-bis(2-methyl-1H-indol-3-yl)-1H-pyrrol-2(5H)-one.

The caveat to this approach however is that changing the maleimide group to a lactone or alternatively a hydroxylactam (not shown) may result in a reduction in enzyme activity. However, the lactam mimics the same type of H-bonding with the hinge region as that some of the carbazole based lactam inhibitors such as K-252c (the staurosporine aglycone). It is possible that the conformationally restricted ring-closed isomer, could also exhibit similar inhibitory effects.
2.6.2 Synthesis of bisindolylmaleimide 2.2

![Scheme 2.20](image)

Scheme 2.20 – Synthesis of 2-methyl bisindolylmaleimide 2.2.

The symmetric bisindole series was prepared in one step using a modified procedure as outlined by Steglich et al. The reaction involves treatment of 2-methylindole with methylmagnesium bromide, followed by slow addition of the dibromomaleimide in a mixture of toluene and THF and heating for several hours. The double addition product was isolated in this one pot procedure in 39 % yield.

2.6.3 Synthesis of bisindolylmaleimide 2.52

Using Sn(0) and HCl, one of the maleimide C=O groups of bisindolylmaleimide 2.2 could be reduced to a CH₂ group. The bisindole lactam was prepared in one step from the bisindolylmaleimide using a Sn/HCl reduction that involved heating the bisindolylmaleimide in ethanol with an excess of tin and concentrated hydrochloric acid, see Scheme 2.21.

![Scheme 2.21](image)

Scheme 2.21 – Synthesis of the lactam 2.52 from the bisindolylmaleimide 2.2.

The lactam product was isolated as a white solid, in 32 % yield. The resulting product is colourless compared to the highly conjugated indolylmaleimide system, which
were deep orange to red in colour. In the $^1$H NMR spectrum, the N-H of the lactam group appears at 8.21 ppm which is expected for an amide. The structure is now asymmetric, two sets of aromatic indole protons, indole N-H and methyl groups are visible. The CH$_2$ group of the lactam cycle appear as two broad 1H singlets centred at 4.41 and 4.48 ppm.

2.6.4 Photochemical properties of 2.2

The changes to the UV-visible absorption spectra of compound 2.2-o with 450 nm light exposure when dissolved in hexanes/THF, THF and DMSO solution are presented in Figure 2.54. In hexanes solution, there are two bands that appear in the visible region, one at 445 nm and a smaller shoulder at ~ 400 nm. With 450 nm light exposure, the band at 445 nm decreases slightly, while the band at 400 nm increases slightly. A new, low intensity band appears at ~553 nm from 2.2-c. The photostationary state is reached after ~7 min (fluorimeter source, 450 nm, 10 nm slit width). Based on the very small changes in the spectrum before and after irradiation, the photostationary state mixture contains mostly 2.2-o.

Similar spectral features were also observed for the THF and DMSO solutions, however the absorption bands are slightly red shifted. The extent of ring-closure was suppressed even more so in THF and DMSO. Though the ring-closed isomers appear stable and do not spontaneously revert back to the ring-open isomer, the extent of photocyclization is low in all solvents to be useful as a photoresponsive inhibitor.
Figure 2.54 – (a) Changes to the UV-visible absorption spectrum of compound 2.2-o, 50 µM when irradiated with 450 nm light at 60 second intervals and dissolved in (a) hexanes/THF (9:1), (b) THF, (c) DMSO (d) Overlap of the changes in absorbance at $\lambda_{\text{max}}$ of the ring-closed isomer as a solution is irradiated with 450 nm light ($\lambda_{\text{max}}$ hexanes = 540 nm, $\lambda_{\text{max}}$ THF = 550 nm, $\lambda_{\text{max}}$ THF = 580 nm).

Compound 2.2-o is highly fluorescent. The intensity of the emission and the emission wavelength is very sensitive to solvent polarity. Like the phenylthiophene series, a strong emission band is observed in non-polar solvents. A representative example of the emission traces of 2.2-o in hexanes/THF (9:1), THF and DMSO are overlaid in Figure 2.55. In the hexanes/THF (9:1) mixture, an intense emission band appears at 554 nm, whereas in THF the emission wavelength is shifted to 572 nm, and
in DMSO the band appears at 603 nm, significantly broadened and intensity diminished substantially.

**Figure 2.55** – (a) normalized excitation and emission spectrum of 2.2-o in hexanes/THF (9:1), (b) Comparison of the changes in the emission wavelength of 2.10-o in solvents of different polarities. Compound 2.2-o, 10 µM in THF/hexanes (9:1), 100% THF, and DMSO.

### 2.6.5 Photochemical properties of 2.52

The UV-visible absorption spectra of compound 2.52-o are very different from the compound described so far in the chapter. The band in the visible region ~440-450 nm observed in the bisarylmaleimides is not present in the lactam derivative 2.52. Instead a single peak appears at ~328 nm which does not change with increasing solvent polarity, see **Figure 2.56 (a-c)**. When a solution of 2.52-o in THF is sequentially irradiated with 330 nm light, two new bands appear in the absorption spectrum over time, a broad band at 532 nm and a less intense band at 388 nm indicating conversion to the ring-closed isomer, 2.52-c. The photostationary state is reached after a total 9 min of irradiation (fluorimeter source, 325 nm, 10 nm slit width). The solution changed from a clear and colourless solution, to pale pink solution when the photostationary state is reached. A similar change is noted for the DMSO solution, although the lower energy peak appears red shifted at 545 nm. The comparable changes in the absorption spectrum of 2.52 in THF and DMSO with light exposure were exciting since the increase in solvent polarity did not appear to influence the photocyclization yield. The lactam modification appears to reduce the compound’s sensitivity to solvent polarity.
Figure 2.56 – UV-visible absorption changes when $2.52$-$o$, 50 µM is irradiated with UV light, 325 nm (fluorimeter source, 10 nm slit widths) in different solvents, (a) THF, (b) DMSO, (c) water (5% DMSO). (d) Overlap of the photostationary state traces of $2.52$ in THF, DMSO, water (5% DMSO), and MOPS buffer (5% DMSO).

Although when the photocyclization was conducted in water or in aqueous buffer, the intensity of the peak in the visible region from the ring-closed isomer $2.52$-$c$ is substantially lower than was observed in DMSO and THF. A small peak appears with exposure of $2.2$-$o$ to 325 nm, at ~525 nm in both water and aqueous buffer. The second higher energy band that appeared at 388 nm in THF and DMSO was absent when irradiation was performed in water or buffer. The photostationary state is reached after ~3 min of irradiation. Further exposure to light, leads to a decrease in the intensity of the band at 525 nm, suggesting that degradation might occur with prolonged irradiation.
2.6.5.1 Ring-opening & stability of lactam 2.52

Figure 2.57 (a) displays the time dependent changes in absorption spectrum of the bisindole lactam switch 2.52 at the photostationary state in an aqueous DMSO mixture (5% DMSO). There is a visible decrease in the intensity of the absorption band at 525 nm from the ring-closed isomer 2.52-c over a period of 1 hr in the dark. The ring-closed isomer 2.52-c is therefore not stable in aqueous solvent, spontaneously reverting back to the ring-open isomer within a few hours at room temperature. The ring-closed isomer 2.52-c behaved differently in THF solution (Figure 2.61 b), since over the same 1 hr time period at room temperature, the intensity of the visible band at 525 nm remains relatively constant.

Figure 2.57 – (a) The ring-closed isomer 2.52-c converts to the ring-open isomer 2.52-o spontaneously in aqueous solvents. (b) Overlap of the time dependent UV-visible absorption spectra from 2.52-PSS, at a concentration of 50 µM in MOPS buffer (5% DMSO), over a 1 hr time period in the dark (right hand side). Changes in the visible absorption band at 525 nm over the 1 hour time period (left hand side). (c) Overlap of the time dependent UV-visible absorption spectra from 2.52-PSS in THF at a concentration of 50 µM, over 1 hour in the dark.

As was mentioned earlier in the chapter (Section 2.3.2.2), the aromatic stabilization energy for indole is higher in comparison to other heterocycles such as
thiophene. Therefore the driving force for the cycloreversion process to convert 2.52-c to 2.52-o

Although it is interesting to note that the cycloreversion process takes places faster in MOPS buffer (Figure 2.57 a) in comparison to THF (Figure 2.57 b). A possible reason for the greater instability of the ring-closed isomer 2.56-c in MOPS buffer could alternatively be due to the change in electronic structure of the π-system after photocyclization. When the ring-closed isomer is formed, aromaticity on the pendant indole heterocycle is disrupted. In the ring-open isomer 2.52-o, the indole nitrogen is relatively non-basic and the lone pair on the indole nitrogen is part of the aromatic ring system. However, in the ring-closed isomer, the indole nitrogen behaves more like a secondary amine and the indole nitrogen can undergo protonation to some extent the aqueous buffer (see Scheme 2.22). Protonation is expected to lower the barrier to cycloreversion and cause spontaneous conversion to 2.52-o. The pKa of the indole nitrogen of the ring-closed cation 2.52-c+ is estimated to be ~4-5, therefore the majority of molecules are expected exist as the non-protonated form, 2.52-c in MOPS buffer (pH 7.2). However, there is a small percentage the electron withdrawing cationic species, 2.52-c+ present in equilibrium with 2.56-c, which could contribute to the faster cycloreversion rate observed in MOPS buffer in comparison to THF. Although further experimentation, looking at the cycloreversion rates in buffers of differing pH are needed to confirm this hypothesis.

![Scheme 2.22 – Possible mechanism for the solvent dependent cycloreversion reaction observed in 2.52-c.](image)

Although the photochemical behaviour initially looked promising in DMSO, we did not investigate the lactam 2.52 further for inhibitory activity against PKC since the
photoconversion yields in aqueous solvents were low and the ring-closed isomer 2.52-c was not kinetically stable.

2.7 Type III – Benzothiophenes

Unfortunately, the results from the last section featuring the bisindole-lactam 2.52 had poor photochemical behaviour. Therefore the bisindole scaffold was ruled out as a possible inhibitor design. In this final series, benzothiophene was chosen as a replacement for one of the indole groups in an effort to mimic the size and the associated geometry of the bisindole as much as possible while simultaneously preserving the photochemical behaviour observed with the thiophene heterocycle.

Figure 2.58 – Representative general structure of the type 3 PKC inhibitor series benzothiophene-indolylmaleimides. The R group is varied from a hydrogen to a N,N'-dimethylaminopropyl group.

The benzothiophene heterocycle shares a number of similar size and shape features as indole, and this ideally will be able to occupy a similar position in the active site as the indole group. Like series 1, one indole and the central maleimide are conserved in the inhibitor design in an effort to maintain the hydrogen-bonding interactions with the amino acids in the hinge region of PKC.

2.7.1 Synthesis of benzothiophene derivatives 2.55 and 2.56.

The synthetic route for the preparation of the target compound is shown in Scheme 2.23. The same Pd(0) catalyzed coupling reaction developed for the earlier phenylthiophene systems was used in the key coupling strategy forming the desired photoresponsive indolylmaleimide benzothiophene core 2.55. Later, the alkyl group was
incorporated using sodium hydride followed by treatment with the corresponding alkyl chloride.

Scheme 2.23 – Proposed synthesis route to access target benzothiophene containing indolylmaleimides 2.55 and 2.56.

Traditionally the maleimide is protected prior to substitution at the indole group. The NaH deprotonation step is presumably unselective, and is expected to occur at both the indole and maleimide groups. However, a recent report by R.-C. Gao et al indicates that this is not always the case\(^{115}\). Maleimide substituted products were not observed under their reported conditions. They speculated that this could be due to formation of the O-alkylated product which later decomposes upon workup with aqueous acid. Gratifyingly, a modified version of this procedure yielded the desired indole substituted product 2.56 was obtained in one step, and the alkylation at the maleimide site was not observed.

The maleimide alkylated product was also prepared using a separate route and included for comparison to compound 2.56. Using relatively mild conditions, compound 2.55 was dissolved in THF and acetonitrile and then mixed with caesium carbonate,
potassium iodide and N′N-dimethylanilinoproylidene hydrochloride at room temperature.\textsuperscript{116}

\textbf{Scheme 2.24} – Synthesis of maleimide alkylated 2.57.\textsuperscript{116}

\textbf{2.7.2 UV-visible absorption properties}

Once the desired product 2.56 was isolated, and its structure characterized, the photochemical behaviour was investigated. Irradiation of a solution of 2.56-\textit{o} in hexanes, 50 \textmu M with 450 nm light (fluorometer source, 10 nm slit width) induces ring-closure to produce 2.56-\textit{c}. This results in the appearance of several new absorption bands, one broad peak centered at 560 nm, two narrower band at 412 nm and 360 nm. A small decrease in the band appearing at 450 nm from the 2.6-\textit{o} is observed as well (see Figure 2.64 (a) below). The solution was irradiated for a total of 10 minutes at 60 second intervals, at which point the photostationary state was reached and no further changes in the absorbance were observed. The isomerization process was accompanied by a colour change, from pale yellow to purple. The ring-closure process was also measured in THF and DMSO. The spectrum of 2.56 in THF before and after irradiation with 450 nm light resembled the results obtained in hexanes solution, although conversion yields were slightly lower in THF, Figure 2.59 (b). Not surprisingly, the conversion yield in DMSO is negligible with almost no noticeable difference in the spectrum before and after irradiation with light. These results demonstrate that the extent of ring-closure, like the thiophene containing series described in the last sections, is highly dependent on solvent medium chosen.
2.7.3 Ring-closed isomer 2.56-c isolation and characterization

To isolate 2.56-c for use in biochemical PKC assays, a solution of 2.56-o (1.4 x 10^{-3} M) in hexanes/THF (1:1) was irradiated with 450 nm light. The 1:1 hexanes/THF mixture was chosen for two reasons, the THF aids in dissolution of 2.56-o, and hexanes was shown to allow for higher photocyclization yields. The progress of the ring-closure reaction was monitored by taking small aliquots of the reaction mixture, and measuring the UV-visible absorption spectrum. Once the photostationary state was reached, (indicated by no further changes in the fluorescence emission spectrum of diluted aliquots taken from the photocyclization reaction mixture), the THF/hexanes was removed under reduced pressure. The residue was purified using column chromatography to yield a deep purple coloured solid, which was diluted in DMSO for studies later on. Due to the sensitivity of 2.56-c to light all manipulations of this compound were performed in the dark.
Figure 2.60 – (a) Conversion of the ring-closed isomer 2.56-c to 2.56-o using 560 nm light (fluorimeter source, 10 nm slit width) induces a colour change from purple to yellow. (b) UV-visible absorption of 2.56-c at 25 µM (pink trace) before and after (dotted black line) exposure to 560 nm light. Irradiation with 450 nm light produces the photostationary state. (c) Cycloreversion kinetics, changes in the absorbance at $\lambda_{\text{max}} = 560$ nm for 2.56-c as a 25 µM solution is irradiated with 560 nm light to produce 2.56-o.

The UV-visible absorption properties of 2.56-c were investigated and the results are summarized in Figure 2.60. In a THF solution of 2.56-c (25 µM), the absorbance spectrum has a broad absorption band at $\lambda_{\text{max}} = 560$ nm, and two narrower bands at 415 nm and 365 nm. Irradiation of the solution with 560 nm light induces cycloreversion to give the ring-open isomer 2.56-o nearly quantitatively. The kinetics of the cycloreversion process is displayed in Figure 2.60 (c), a plot of the absorbance at 560 nm over time shows a dramatic decrease in the intensity. Cycloreversion is complete within ~4 min. under these irradiation conditions (fluorimeter source, 560 nm light, 10 nm slit width). However the rate of conversion of 2.56-c to 2.56-o can be tuned by adjusting the
wavelengths and power of the light source. For example, using a tungsten lamp source (150 W) with a >490 nm cut-off filter induces the cycloreversion process within a few seconds (data not shown).

The photostationary state for the ring-closing reaction (conversion of 2.56-o to 2.56-c) was estimated using the data obtained from the absorption plot in Figure 2.60 (b). The concentration of ring-closed isomer in the photostationary state can be estimated using Beer’s law. The extinction coefficient of the pure ring-closed isomer trace 2.56-c, at $\lambda_{\text{max}} = 560$ nm is $\varepsilon = 8,800$ cm$^{-1}$ M$^{-1}$ for 2.56-c (25 µM THF). Using the absorbance value at 560 nm in the photostationary state, the concentration of ring-closed isomer is calculated to be 6.8 µM, and the photostationary state is estimated to be 27%.

2.7.4 Characterization of 2.56-c and 2.56-o with $^1$H NMR spectroscopy

The $^1$H NMR of the isolated ring-closed isomer 2.56-c was obtained in DMSO-d$_6$ and the aromatic region is shown in Figure 2.61 along with the ring-open isomer 2.56-o. The ring-open isomer was generated by irradiation of the NMR tube with >490 nm light. Comparison of the aromatic region of the ring-closed and ring-open isomers, the most significant differences are observed in the indole and benzothiophene protons labelled 2 and 6 in 2.56-o (and labelled 2’ and 6’ in 2.56-c). In 2.56-c, these resonances appear at 9.25 ppm (for 2’) and 8.63 ppm (for 6’), and in 2.56-o they appear at 7.82 and 7.37 ppm. The 2’ and 6’ doublets become more deshielded as the molecule is forced into a nearly planar conformation, and the protons are within close proximity to the maleimide carbon oxygen double bond. This pattern was also observed in 2.10-c described for the thiophene series earlier.

Interestingly, several of the aromatic resonances of the 2.56-o are not well resolved and appear as broadened peaks. The integration of the broadened regions match the expected values, a total of 8 aromatic protons. Based on chemical shift, and coupling constants, the aromatic protons nearest the indole nitrogen are likely poorly resolved due to slow exchange of the two aryl residues from a number of possible structures. In the ring-closed isomer, 2.56-c all of the peaks are well resolved, which is a
reasonable observation considering that the aryl groups are conformationally restricted. The unsubstituted indole precursor, 2.55-o does not show the same peak broadening that is observed with 2.56-o suggesting the slow exchange is unique to the alkylated indole product.

![Chemical Structure](image)

**Figure 2.61** – Partial $^1$H NMR spectrum of compound 2.56-c (top) and 2.56-o (bottom) in DMSO-d$_6$, 400 MHz.

The maleimide substituted compound 2.57, was also included for comparison and to help aid in the characterization of 2.56. The $^1$H NMR spectrum of 2.57-c and 2.57-o in DMSO-d$_6$ are overlapped in **Figure 2.62**. A dramatic change in the chemical shift of the indole nitrogen was observed for compound 2.57 with ring-closure. In the ring-open isomer, the indole proton appears at 11.50 ppm, an expected region. Although in the
ring-closed isomer, 2.57-c the indole N-H is shifted upfield ~4.57 ppm to 6.93 ppm. This change in chemical shift reflects the differences in the electronic structure of the indole \( \pi \)-system with ring-closure. Photocyclization leads to disruption of aromatization and the indole nitrogen has secondary amine characteristics.

![Chemical structures and NMR spectra](image)

**Figure 2.62** – Partial \(^1\)H NMR spectrum of compound 2.57-o (top) and 2.57-c (bottom) in DMSO-d\(_6\), 400 MHz.

### 2.7.5 Solubility

The stability of 2.56 was evaluated in HEPES buffer using a combination UV-visible absorption spectroscopy and DLS to check for the presence of aggregate species. The results from the study are summarized in Figure 2.63.
Figure 2.63 – (a) Time dependent changes in the absorption spectrum of a solution of 25 µM ring-closed isomer 2.56-\textit{c} in HEPES buffer, pH 7.5, 1% DMSO monitored over 2 hours at 25°C, in the dark. Sequential measurements were made every 10 minutes and overlapped for comparison. (b) The changes in the absorption spectrum of a solution of 25 µM ring-open isomer 2.56-\textit{o} in HEPES buffer, pH 7.5, 1% DMSO, monitored over 2 hours in HEPES buffer, in the dark. Sequential measurements were made every 10 minutes and overlapped for comparison. The inset shows a zoom in of the absorption band at ~450 nm. (c) Summary of changes to UV-visible absorption spectra of 2.56-\textit{c} at 560 nm (pink circles) and 2.56-\textit{o} at 455 nm (orange hollow circles) each over a two hour period. (d) Images taken of the cuvette at different time points in the experiment.

The absorption spectrum of 2.56-\textit{c} in HEPES buffer plotted in Figure 2.63 (a) shows a remarkable decrease in the intensity of the absorption trace over the 2 hr time period that the measurements were taken. The bands at $\lambda_{\text{max}} = 560$ nm, 421 nm, and 365 nm decreased in intensity $\sim 65\%$ during the 2 hr incubation period in buffer. This indicates that the ring-closed isomer is not stable in buffer system and likely forms aggregate particles which sediment out of solution. Interestingly, at the start of the experiment, the solution appeared clear and pink in colour with no visible particles or cloudiness, see Figure 2.63 (d) for image. Inspection of the solution in the cuvette 2 hrs later, the sample appeared much paler in colour, nearly clear.
Under a bright light, small red coloured aggregate particles suspended in solution were visible after incubation in aqueous buffer, see Figure 2.64 (b). The cuvette image shown in Figure 2.64 (a), was taken at the start of UV-visible experiment. Here the solution appears clear, pale purple/pink coloured. Exposure of the aggregate suspension in the cuvette to >490 nm light for a few seconds led to generation of the ring-open isomer, 2.56-o, Figure 2.64 (c), which appears as a yellow coloured aggregate suspension. Continued irradiation with >490 nm light for ~60 seconds, led to dissolution of the aggregates, Figure 2.64 (d). The solution returned to the expected yellow colour, clear and the aggregate particles were no longer visible.

![Figure 2.64](image)

**Figure 2.64** – (a) Image taken shortly after the preparation of a 25 µM solution of 2.56-c in HEPES. (b) Image of the cuvette containing 2.56-c solution in HEPES buffer after 2 hr in the dark at RT. Viewed under a bright light source, a red coloured aggregate suspension is visible. (c) Exposure of the cuvette to >490 nm light produces 2.56-o. (d) 2.56-o is soluble in the HEPES buffer medium, and the aggregates dissolve within a few seconds to give a clear, yellow solution.

A solution of 2.56-c in HEPES buffer was sandwiched between two glass slides and viewed under a microscope (see Figure 2.56 a-d). The aggregates appeared as irregularly shaped clusters of differing sizes.
Attempts to dissolve the aggregates with light exposure when sandwiched between the glass slides were not successful, and remained visible under the microscope with 490 nm light irradiation.

Small molecule inhibitor candidates that form aggregate species in solution are undesirable for targeting enzymes in vitro and in vivo due to their lack of selectivity and unpredictable concentration. However compound 2.56 exhibits light dependent aggregation, with only particle formation observed in the ring-closed state 2.56-c. The aggregates can be re-dissolved on command by a few seconds of irradiation with yellow light to produce the 2.56-o which is stable in aqueous media. Therefore we hypothesized that 2.56 may still serve as an interesting photoresponsive inhibitor candidate if the aggregate particles can inhibit aggregate induced inhibition reversibly.

The ring-opening kinetics of 2.56-c were investigated in HEPES buffer (pH 7.5) to determine ideal conditions for generation 2.56-o. When a 25 µM solution of 2.56-c is irradiated with 560 nm (fluorimeter source, 10 nm slit width, ~1 mW/cm²), the ring-open isomer 2.56-o is generated after ~7 min of light exposure, Figure 2.66 (a). However,
during the ring-opening process, the absorption traces shift slightly with sequential exposure to 560 nm light. There is also an increase in the absorption between 650-800 nm. These changes suggest that the solution is beginning to form aggregates during the time it took to complete the experiment. A second experiment was performed using the same 25 µM solution of 2.56-c, however the light used to induce cycloreversion was changed to a more intense source (>490 nm filter, 37 mW/cm²). The ring-opening process is near completion after a much shorter time, ~10-20 sec of light exposure. The overlapped spectral traces collected (displayed in Figure 2.66 b) suggest that aggregate formation did not occur in this shorter time period.

![Chemical structures of 2.56-c and 2.56-o](image)

**Figure 2.66** – (a) Overlapped changes to the UV-visible absorption spectrum of a 25 µM solution of 2.56-c (HEPES pH 7.5, 1% DMSO) sequentially exposed to 560 nm light (at 30 second intervals until 5 minutes and then 60 second intervals onward). (b) Overlapped changes to the UV-visible absorption spectrum of a 25 µM solution of 2.56-c (HEPES pH 7.5, 1% DMSO) exposed to >490 nm filter source (10 second irradiation intervals). (c) Plot of the absorbance of 2.56-c at $\lambda_{\text{max}}$ 560 nm as it is irradiated with > 490 nm light to produce 2.56-o.

**2.7.6 Stability of 2.57-c in aqueous buffer**

Interestingly, compound 2.57-c did not exhibit the same self-assembly properties as 2.56-c. The two structures differ in the position of the N,N'-dimethylaminopropyl
chain. Both isomers of 2.57 appeared to be stable in aqueous buffer over the 2 hr time period, see Figure 2.67 (a-d) below.

![Graphs](image)

**Figure 2.67** – a) Time dependent changes in the absorption spectrum of a solution of 50 µM ring-closed isomer 2.57-c in MOPS buffer, 1% DMSO monitored over 2 hours at 25°C, in the dark. Sequential measurements were made every 5 minutes (only 10 minute traces are shown) and overlapped for comparison. (b) Summary of changes in the absorption at $\lambda_{\text{max}}$ 532 nm and 412 nm over time. (c) Overlapped absorption spectrum of a solution of 25 µM ring-open isomer 2.57-o in MOPS buffer, 1% DMSO, monitored over 2 hours in HEPES buffer, in the dark. Sequential measurements were made every 5 minutes and overlapped for comparison. The inset shows a zoom in of the absorption band at $\sim$465 nm. (c) Summary of changes in the absorption at $\lambda_{\text{max}}$ 465 nm over time.

In 2.57-c, the maleimide is substituted with the aminoalkyl group, whereas in 2.56-c the indole is substituted. Compounds 2.57-c and 2.56-c can adopt a somewhat planar conformation with limited rotational freedom. This could increase the likelihood that assemble into aggregates in aqueous solution. However, 2.56-c was clearly more
prone to aggregation than 2.57-c. The free maleimide group in 2.56-c likely serves as a template for hydrogen bond formation, accelerating interactions with other molecules in solution, and increasing the rate of aggregation formation. Efficiency

2.7.7 Enzyme activity

2.7.7.1 In vitro PKC β-II inhibitory assay screen

The inhibitory activity of the photoresponsive derivatives synthesized were investigated against PKC β-II, using the FRET peptide procedure described in Section 2.5 of this chapter. Compounds 2.55-o, 2.56-o and 2.2-o were screened at a concentration of 1 µM in (1% DMSO), and the percentage inhibition that was measured during the 30 minute phosphorylation reaction is summarized in Table 2.11.

Table 2.11 – Summary of activity for selected compounds 2.55-o, 2.56-o, 2.2-o and selected compounds from the phenylthiophene series against PKC β-II

<table>
<thead>
<tr>
<th>Compound</th>
<th>% Inhibition at 1 µM (phosphorylation % relative to DMSO control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2-o</td>
<td>45</td>
</tr>
<tr>
<td>2.55-o</td>
<td>20</td>
</tr>
<tr>
<td>2.56-o</td>
<td>19</td>
</tr>
</tbody>
</table>

The study revealed interesting results. The benzothiophene containing inhibitors gave only a moderate reduction in enzyme activity, under the assay conditions. The N-H indole 2.55-o, reduced activity 20%, relative to the 1% DMSO control. Unfortunately, the N,N'-aminopropyl substituted indole 2.56-o, had a very similar reduction in activity (19 % versus control). The results were surprising, considering the similarities between 2.56-o and the bisindolylmaleimide based inhibitors, for example GF 109203X which demonstrated nearly complete abolishment of activity at 1 µM (demonstrated in Section 2.5.6.2). The 2-methyl-BIM 2.2-o gave moderate inhibition with a 45% reduction in activity. The data suggest that the presence of the benzothiophene instead of two indole groups is problematic for the inhibitor design.
Despite the moderate inhibition of 2.56-o, the activity of PKC could be modulated with light by taking advantage of the light controlled aggregation properties. Aggregate induced inhibition is typically dependent on the concentration of enzyme in solution, however reversible inhibition of enzyme function via the aggregate induced inhibition mechanism would be an interesting proof of concept study. The mechanism by which inhibition is achieved involves physical adsorption or sequestration of enzyme on the surface of the particles. Compound 2.56 could be a useful tool to investigate whether or not inhibition of enzyme activity by an aggregate induced mechanism can be reversed. As well as to investigate whether or not full restoration of enzyme activity is possible simply by dissolving aggregate species.

2.7.7.2 Light controlled inhibitory activity

The activity of 2.56-o was investigated further, to determine its dose-response behaviour. Concentration was varied from 25 µM to 0.05 µM and then assayed in duplicate using the same conditions described for the screening study in Section 2.5.6.2. The data was fitted to a dose-response curve (using the variable slope, four parameter function in Prism, version 7.0, GraphPad Software Inc, 1995-2016). The estimated IC<sub>50</sub> was 4.5 µM for compound 2.56-o and a Hill slope of 1.3. The dose-response curve obtained during the assay is shown below in Figure 2.68.

![Figure 2.68](image-url)

**Figure 2.68**– Log of the inhibitor concentration (µM) versus the percentage relative activity of the control sample, (1% DMSO). Compounds 2.56-o (orange trace), 2.56-c (pink trace) and 2.56-c + exposure to yellow light (>490 nm cut-off filter) (purple trace). Error bars are mean ± standard deviation of duplicate samples.
A second assay was conducted to include the ring-closed isomer \textit{2.56-c} and \textit{2.56-c} exposed to yellow light (>490 nm filter) for 30 seconds prior to measurement in the assay. In the micromolar range, (concentration of 25-1.25 µM), \textit{2.56-c} inhibited the kinase activity almost completely, with very little to no phosphorylation at these concentrations during the assay. This is presumably due to the aggregate species readily formed in the assay buffer used. As the concentration was decreased, the inhibitory activity became less effective, and a rapid increase in the kinase activity is observed at concentrations >0.2 µM. The sharp increase in activity is expected for aggregate-induced inhibition, possibly due to the absence of aggregates at this low concentration.

Exposing \textit{2.56-c} (at 625 nM) to >490 nm light for 30 seconds prior to initiating the assay, resulted in ~30 % reduction in kinase activity relative to the control (purple trace in \textbf{Figure 2.72}). At the same concentration, the ring-closed isomer \textit{2.56-c} reduced enzyme activity by ~85%, therefore the results suggest that light irradiation allows for efficient restoration of the ring-open isomer in solution. However, the activity measured for \textit{2.56-c} + >490 nm light, does not overlap with the curve generated for pure \textit{2.56-o}. This could be due to several reasons, one possibility being that dissolution of aggregate species \textit{in situ} may not restore the enzyme’s activity completely. Binding to aggregates could, for instance permanently denature a percentage of the enzyme. However, further studies are required to evaluate the nature of the aggregate and enzyme interactions.

\textbf{2.8 Chapter summary and conclusions}

Overall, Chapter 2 demonstrated the concept of using light induced geometry changes to influence the activity of a protein kinase through two different mechanisms; one relying on changes to the steric or physical orientation of key pharmacophoric groups to influence active site binding affinity, and the second using differences in the ability of a small molecular inhibitor to self-assemble into aggregate structures which exert their inhibitory effects through non-specific sequestration and adsorption of enzyme onto its surface.
In the course of designing a practical and useful photoresponsive protein kinase inhibitor with a bisindolylmaleimide core, several obstacles hindered progress. The most challenging problem to overcome, was the selection of an appropriate derivative that would maintain ideal photochemical behaviour, while retaining enzyme inhibitory activity, and stability in aqueous media. The presence of the indolyl-maleimide, essential for binding affinity, also severely hinders photochemical switchability, especially in polar solvents.

A number of thiophene based inhibitors were studied first, these contained either a phenyl substituent (series 1-A) or an alkyl substituent (series 1-B). Photoresponsive behaviour was moderate to excellent in non-polar solvents, depending on the substituents present on the phenyl ring. However, all had poor photochemical ring-closure yields in aqueous buffer. Unfortunately, the inhibitory activity of the phenylthiophene derivatives were all considerably poor, with <10% inhibition at 1 µM. The alkylthiophene derivative 2.10-o, demonstrated the most potent enzyme inhibition, with a measured IC$_{50}$ of 0.58 µM. The ring-closed isomer 2.10-c, did not have much of an effect on activity, with an IC$_{50}$ >25 µM, predicted during our study. This particular derivative also proved to be useful for unidirectional light activated inhibition. The pure inactive isomer, 2.10-c was exposed to visible light for 30 seconds, to generate the active isomer 2.10-o. The dose-response curves of the cycloreversion product, resembled that of pure 2.10-o, indicating that complete conversion had taken place from the inactive to the active state. Unfortunately, our system only allowed for rapid conversion from the 'off' state, (inactive inhibitor) to the 'on' state (active inhibitor) using light, since reversibility of the activity was not possible, due to the poor photocyclization yield in polar solvents.

The lactone derivatives featured in the type 2 inhibitor design, allowed for the modification of the electronic structure of BIM core and were investigated as an alternative to overcome the poor photostationary state observed in polar solvents. The findings from the UV-visible absorption measurements, indicated that the photochemical ring-closure process was less sensitive to solvent polarity when the central maleimide is replaced with a lactone group. Photocyclization yields were comparable in DMSO and THF, which was a substantial improvement over the type 1 inhibitors. However, we
noticed that spontaneous conversion of the ring-closed isomer to the ring-open isomers occurred in water and in aqueous buffer within several hours. The indole nitrogen is more basic in the ring-closed state, because the lone pair on the nitrogen atom is not part of the $\pi$-system and can be protonated in water. The resulting cationic species, is less stable and lowers the cycloreversion barrier to ring-opening. Due to the poor thermal stability of the type 2 lactone derivatives, these were not included in kinase activity studies.

Overall the biochemical activity data and behaviour of the benzothiophene derivative described in this section were surprising. The benzothiophene substituent was included in the inhibitor structure in attempts to mimic the shape of the BIM structure more closely. Unfortunately, the target compound 2.56-o resulted in a substantial loss in activity, with a predicted IC$_{50}$ of 4.5 $\mu$M, which is 200-fold higher than the IC$_{50}$ for the indole containing compound 2-methyl-BIM (Table 2.2). Contrary to our expectations, the ring-closed isomer 2.56-c demonstrated higher inhibitory activity against PKC. The stability of the ring-closed isomer is limited in aqueous solution and begins to spontaneously aggregate into micron sized particles after <2 hr. The aggregate species appeared to inhibit the enzyme activity nearly completely at micromolar concentration ranges via an aggregate induced inhibition mechanism. It should be noted that aggregate induced inhibition, is dependent on the concentration of enzyme in solution, so this type of inhibition usually has limited use in biological assays.

There are several of factors that are not suitably addressed with this technology, that may limit the range and type of applications possible. A main concern is that although there was a measurable difference in the activity of the two photoisomers, (as shown for 2.10), during the biochemical assays against purified PKC $\beta$-II, the off-target activity of both isomers was not evaluated and remains unknown. The ring-closed isomer, 2.10-c, is referred to as the “inactive” isomer, however its activity was only tested against one isoform of PKC. Therefore, its activity should be further tested against a wider panel of biological targets, this cannot be referred to as a completely inactive compound.
2.9 Future work

Investigate the light induced aggregate inhibition. The inhibition is non-specific could be applied as a general light controlled “inhibitor” for a range of enzymes. Further work could be done to evaluate how aggregate based systems perform as inhibitors. The light controlled aggregate solubility could be applied as a tool and determine whether or not their effects are fully-reversible or dependent on time, concentration, additives etc.

The mechanism of inhibition for alkylthiophene derivative 2.10-o was assumed to be ATP competitive. Although further examination of the inhibitory activity at varying ATP concentrations is required to confirm or refute this.

2.10 Experimental Section

2.10.1 General.

All solvents and reagents used for synthesis, chromatography, UV-vis spectroscopy and photochemical studies were purchased from Aldrich, Anachemia, Caledon Labs, Fisher Scientific or Alfa Aesar. Unless otherwise noted, all solvents used for synthesis were dried and degassed by passing them through steel columns containing activated alumina under nitrogen using an MBraun solvent purification system. Solvents for NMR analysis were purchased from Cambridge Isotope Laboratories and used as received. Column chromatography was performed using silica gel 60 (230–400 mesh) from Silicycle Inc.

2.10.2 Instrumentation.

$^1$H and $^{13}$C NMR characterizations were performed on a Bruker Avance-400 instrument with a 5 mm inverse probe operating at 400.13 MHz for $^1$H NMR and 100.61 MHz for $^{13}$C NMR. Chemical shifts ($\delta$) are reported in parts per million relative to tetramethylsilane using the residual solvent peak as a reference. Coupling constants ($J$) are reported in Hertz. UV-visible absorption spectra were recorded on a Varian Cary 300
Bio-spectrophotometer. High Resolution Mass Spectroscopy (HRMS) measurements were performed using an Agilent 6210 TOF LC/MS in ESI-(+) mode. Melting Points were measured using a Fisher-Johns melting point apparatus, or a Gallenkamp melting point apparatus (Registered Design No. 889339).

2.10.3 Photoinduced ring-closing and ring-opening reactions.

Irradiation with monochromatic light was generated using the source from a PTI Quantamaster Spectrofluorimeter and was used to carry out the ring-closing reactions. 10 nm slit width was used in all cases unless otherwise mentioned. Irradiation with visible light was carried out using the light of a 150-W tungsten source that was passed through a 490-nm cut-off filter to eliminate higher-energy light. Irradiation was carried out in dark conditions to minimize interference from ambient light.

2.10.4 In vitro enzyme assay

Recombinant full-length human protein kinase C βII expressed by baculovirus in insect Sf9 cells using a N-terminal GST tag were purchased from SignalChem, (P63-10G SignalChem, Richmond, BC, Canada). Phosphorylation activity was evaluated using a Z'-Lyte ratiometric FRET-based kinase assay kit according to the manufacturer’s instructions (PV3180 Invitrogen Corporation, Carlsbad, CA, USA). Briefly, in the first stage of the assay, the protein kinase was incubated with a synthetic peptide substrate labelled with a coumarin donor and a fluorescein acceptor FRET pair. In the non-phosphorylated state, the peptide was cleaved in the second development stage of the assay disrupting the FRET pair, and giving rise to an increase in coumarin emission. In the phosphorylated state, the presence of the phosphate group suppresses peptide cleavage and the FRET process dominates. The phosphorylation reaction progress was calculated from the ratio of the measured coumarin emission to fluorescein emission. Kinase assays were performed in uncoated, black, flat bottom 96 well plates. PKC βII (0.5 μg/mL) was treated at room temperature for 1 h with Z'-Lyte Ser/Thr 7 peptide (2 μM), ATP (25 μM) and with either inhibitor samples at various concentrations or with DMSO control (1%). The assay buffer contained HEPES buffer (25 mM, pH 7.5), MgCl₂ (10 mM), EGTA (1 mM), 0.01% Brij-35 and an activator mixture with phosphatidylserine.
(12.5 µg/mL), 1-stearoyl-2-linoleoyl-sn-glycerol (1.2 µg/mL) and 1-oleoyl-2-acetyl-sn-glycerol (1.2 µg/mL). The activator mixture was sonicated for 60 s prior to adding it to the assay buffer. The development reagent was added to the wells and incubated at room temperature for 1 h followed by the stop solution. The degree of phosphorylation was calculated by measuring the emission ratio of coumarin (λ<sub>exc</sub> = 400 nm, λ<sub>em</sub> = 445 nm) and fluorescein (λ<sub>exc</sub> = 400 nm, λ<sub>em</sub> = 520 nm) for each well. Emission ratios were measured on a BioTek Synergy™4 Hybrid Microplate Reader.

2.10.5 Synthetic Procedures

2.10.5.1 Synthesis of 1,2-dimethylindole.

Prepared based on literature procedure.<sup>117</sup> A solution of 2-methylindole (2.37 g, 1.81 x 10<sup>-2</sup> mol) in anhydrous DMF (25 mL) was added drop wise via a syringe to a cooled (~0°C ice bath) suspension of sodium hydride (anhydrous, 0.48 g, 2.00 x 10<sup>-2</sup> mol) in anhydrous DMF (50 mL). After the addition was complete, the reaction mixture was stirred at 0°C for an additional 20 min, followed by the drop wise addition of methyl iodide (1.2 mL, 1.99 x 10<sup>-2</sup> mol). After the addition was complete, the cooling bath was removed, allowed to reach room temperature and stirred for 18 h. The mixture was carefully quenched with a solution of NH<sub>4</sub>Cl (10 mL), the contents of the flask were poured into 100 mL of water and extracted with EtOAc (2 x 100 mL). The organic layers were combined and dried over MgSO<sub>4</sub>, filtered and then concentrated under reduced pressure. The resulting brown oil was purified using column chromatography (silica, 20% EtOAc / hexanes) to yield 2.2 g of a pale yellow powder, 84%.

<sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 7.58 (d, J = 7.8 Hz, 1H), 7.32 – 7.29 (m, 1H), 7.23-7.19 (m, 1H), 7.16 – 7.10 (m, 1H), 6.31 (s, 1H), 3.71 (s, 3H), 2.48 (s, 3H).
2.10.5.2 Synthesis of Ethyl 2-(1,2-dimethyl-1H-indol-3-yl)-2-oxoacetate

Prepared based on literature procedure. A room temperature solution of 1,2-dimethylindole (1.99 g, 1.37 x 10^{-2} mol) in anhydrous ether (50 mL) was treated with oxalyl chloride (1.74 mL, 9.13 x 10^{-3} mol) drop wise over 10 min. This solution was kept at room temperature for 1 h and was then cooled to -60ºC and a solution of sodium ethoxide (9.9 mL, 21% wt in EtOH) was added drop wise via a syringe. The suspension was stirred for 30 min at -60ºC and then allowed to warm to RT. The reaction mixture was diluted with EtOAc, washed with NaHCO₃ (sat’d) followed by water. The organic layer was dried over MgSO₄, filtered and concentrated to dryness, yielding yellow solid. The solid was triturated with Et₂O to give white crystals, 2.4 g, 71%.

¹H NMR (500 MHz, CDCl₃) δ 7.96 – 7.90 (m, 1H), 7.30 – 7.24 (m, 3H), 4.48 (q, J = 7.2 Hz, 2H), 3.64 (s, 3H), 2.63 (s, 3H), 1.44 (t, J = 7.2 Hz, 3H).

¹³C NMR (126 MHz, CDCl₃) δ 181.79, 166.37, 147.77, 136.88, 126.03, 123.14, 123.05, 123.00, 122.89, 120.33, 120.09, 109.79, 109.72, 109.70, 109.63, 109.39, 61.91, 29.72, 13.97, 12.36.

2.10.5.3 Synthesis of methyl 2-(2-methyl-1H-indol-3-yl)-2-oxoacetate

2-methylindole (1.53 g, 1.17 x 10^{-2} mol) was dissolved in diethyl ether (anhydrous, freshly distilled). Next, oxalyl chloride (1.4 mL, 1.63 x 10^{-2} mol) was added to the solution drop wise via a syringe at room temperature. After the addition was complete, the resulting solution was stirred under nitrogen protection at room
temperature for 2 h. The mixture was cooled to 0 °C (ice/water bath) and then treated drop wise with sodium methoxide (25 wt % solution in methanol, 7.1 mL, 3.27 x 10⁻² mol). After the addition was complete, the mixture was kept at 0 °C for an additional 15 min and then allowed to warm to RT gradually. The reaction progress was monitored by TLC and upon consumption of the starting materials, the flask contents were poured into a separatory funnel extracted with EtOAc (2 x 50 mL). The organic layers were combined and washed with sat’d NaHCO₃ (50 mL) followed by water (50 mL). The organic layers were dried over MgSO₄, filtered, and concentrated to dryness under reduced pressure. The orange/brown solid was triturated with diethyl ether, to give a pale yellow, fine granular powder 1.98 g (78 %).

¹H NMR (500 MHz, Acetone) δ 7.99 (dd, J = 6.4, 2.6 Hz, 1H), 7.50 – 7.40 (m, 1H), 7.28 – 7.18 (m, 2H), 3.98 (s, 3H), 2.66 (s, 3H).

### 2.10.5.4 Synthesis of 2-methyl-5-phenylthiophene-3-carbaldehyde

Prepared based on literature procedure.¹⁰⁰ A solution of 3-bromo-2-methyl-5-phenylthiophene (3.2 g, 1.26 x 10⁻² mol) in anhydrous THF (~100 mL) was treated at -60°C with n-BuLi (8.6 mL, 1.39 x 10⁻² mol) over a 20 min period. The reaction was stirred at -60°C for an additional 30 min and was then quenched with DMF (anhydrous, 1.96 mL, 2.53 x 10⁻² mol). The mixture was kept at -60°C for 30 min and afterwards, the cooling bath was removed and the flask gradually allowed to come to RT. An aqueous solution of HCl (2 M, 100 mL) was added to the flask and the flask contents were poured into a separatory funnel, followed by extraction with Et₂O (2 x 100 mL). The combined organic layers were then washed with brine, water and then dried over MgSO₄, filtered and concentrated under reduced pressure to yield a fluffy, pale yellow solid, 2.39 g 93 %.

The material isolated in this step was used without further purification.
1H NMR (500 MHz, CDCl\textsubscript{3}) \(\delta\) 10.03 (s, 1H), 7.58 – 7.55 (m, 3H), 7.41 – 7.37 (m, 2H), 7.33 – 7.29 (m, 1H), 7.26 (s, 2H), 2.81 (s, 3H).

2.10.5.5 Synthesis of (2-methyl-5-phenylthiophen-3-yl)methanol

\[
\begin{align*}
\text{2-methyl-5-phenylthiophen-3-carbaldehyde} & \xrightarrow{\text{NaBH}_4, \text{MeOH}} \text{(2-methyl-5-phenylthiophen-3-yl)methanol} \\
\end{align*}
\]

Prepared based on literature procedure.\textsuperscript{101} A solution of 2-methyl-5-phenylthiophene-3-carbaldehyde (1.58 g, 7.81 \times 10\textsuperscript{-3} mol) in 100 mL of methanol was cooled to 0\textdegree C with an ice/water bath. Sodium borohydride (0.59 g, 1.56 \times 10\textsuperscript{-2} mol) was added to the flask in portions over \sim 10 min. The cloudy suspension was stirred for 1 hour at 0 \textdegree C and was then poured into 100 mL of 5\% \text{H}_2\text{SO}_4 (aq) and extracted with \text{Et}_2\text{O} (2 \times 50 mL), washed with brine and water. The organic layers were combined, dried over \text{MgSO}_4, filtered and concentrated under reduced pressure to give opaque yellow flakes, 1.28 g (80\%). The material was used in the next step without further purification.

1H NMR (500 MHz, CDCl\textsubscript{3}) \(\delta\) 7.57 – 7.51 (m, 2H), 7.38 – 7.32 (m, 2H), 7.25 – 7.23 (m, 1H), 7.22 (s, 1H), 4.61 (s, 2H), 3.49 (s, 1H), 2.47 (s, 3H).

2.10.5.6 Synthesis of 3-(bromomethyl)-2-methyl-5-phenylthiophene

\[
\begin{align*}
\text{(2-methyl-5-phenylthiophen-3-yl)methanol} & \xrightarrow{\text{PBr}_3, \text{CHCl}_3} \text{(3-(bromomethyl)-2-methyl-5-phenylthiophene)} \\
\end{align*}
\]

Prepared based on literature procedure.\textsuperscript{101} To a 250 mL RBF was added (2-methyl-5-phenylthiophene-3-yl)methanol (2.59 g, 1.27 \times 10\textsuperscript{-2} mol) followed by 100 mL of \text{CHCl}_3. While stirring under nitrogen, the resulting solution was treated drop wise with \text{PBr}_3 (2.38 mL, 2.54 \times 10\textsuperscript{-2} mol) over \sim 5 min period. The solution was left to stir at RT overnight. Afterwards it was poured into water, and extracted with \text{Et}_2\text{O} (2 \times 100 mL).
The combined ether layers were washed with NaHCO₃ (50 mL), followed by water (50 mL), and then dried over MgSO₄, filtered and concentrated under reduced pressure to give pale yellow flakes 1.32 g (39%).

\[\text{H NMR (500 MHz, CDCl}_3\text{)} \delta 7.55 - 7.52 (m, 2H), 7.38 - 7.33 (m, 2H), 7.29 - 7.25 (m, 2H), 7.18 (s, 1H), 4.47 (s, 2H), 2.46 (s, 3H).\]

**2.10.5.7 Synthesis of 2-(2-methyl-5-phenylthiophen-3-yl)acetonitrile**

Prepared based on literature procedure.¹⁰¹ To a 50 mL RBF was added 3-(bromomethyl)-2-methyl-5-phenylthiophene (1.30 g, 4.87 x 10⁻³ mol) followed by 5 mL of benzene, 10 mL of water and stirred to dissolve. NaCN (0.48 g, 9.73 x 10⁻³ mol) and tetrabutylammonium bromide (0.08 g, 2.48 x 10⁻⁴ mol) were then added and the mixture was heated to reflux overnight. The next day, the heat was removed, and the reaction flask allowed to cool to RT. After which, the reaction mixture was poured into a separatory funnel and extracted with CHCl₃. The organic layer was washed with brine and water, dried over MgSO₄, filtered and concentrated under reduced pressure. The resulting crude brown oil was purified using column chromatography (25% EtOAc / hexanes, silica gel) to yield a yellow crystalline solid, 0.75 g, 72%.

\[\text{H NMR (400 MHz, CDCl}_3\text{)} \delta 7.59 - 7.53 (m, 2H), 7.43 - 7.35 (m, 2H), 7.34 - 7.27 (m, 1H), 7.18 (s, 1H), 3.62 (s, 2H), 2.45 (s, 3H).\]

\[\text{C NMR (101 MHz, CDCl}_3\text{)} \delta 141.39, 135.54, 133.87, 129.05, 127.72, 125.87, 125.60, 123.84, 123.83, 117.55, 17.08, 13.26.\]

FT-IR (solid sample) = 2915, 2246, 1500, 1443, 760 cm⁻¹.

2.10.5.8 Synthesis of 2-(2-methyl-5-phenylthiophen-3-yl) acetic acid

![Chemical structure](image1)

Prepared based on literature procedure.\textsuperscript{101} To a 25 mL RBF was added 2-(2-methyl-5-phenylthiophen-3-yl)acetonitrile (0.67 g, 3.14 \times 10^{-3} \text{ mol}) followed by 10 mL HCl (concentrated, \~37\%). The suspension was heated to reflux for \~4 hr. Next the reaction was allowed to cool to RT, after which the mixture was poured into CHCl\textsubscript{3} (100 mL). The brown/cloudy CHCl\textsubscript{3} layer was collected and washed with NaHCO\textsubscript{3} (sat’d), followed by water, then dried over MgSO\textsubscript{4}, filtered and concentrated under reduced pressure. Yielding a pale yellow powder, 0.65 g, 89 %.

\textsuperscript{1}H NMR (500 MHz, CDCl\textsubscript{3}) \delta 7.55 – 7.51 (m, 2H), 7.36 – 7.32 (m, 2H), 7.26 – 7.22 (m, 1H), 7.12 (s, 1H), 3.59 (s, 2H), 2.42 (s, 3H).

2.10.5.9 Synthesis of 2-(2-methyl-5-phenylthiophen-3-yl) acetamide

![Chemical structure](image2)

To a 100 mL RBF containing 2-(2-methyl-5-phenylthiophen-3-yl) acetic acid (0.63 g, 2.72 \times 10^{-3} \text{ mol}) was added CHCl\textsubscript{3} (50 mL) followed by thionyl chloride (0.80 mL, 1.09 \times 10^{-2} \text{ mol}). The solution was heated to reflux for 1 hr, the heating source removed and was then allowed to cooled to RT. Afterwards, the RT solution was treated with an excess of NH\textsubscript{4}OH solution, 28\% v/v in water (5.26 mL, 4.1 \times 10^{-3} \text{ mol}) and stirred for 2 hr. The precipitate that formed was filtered using a Hirsch funnel, washed with water and then allowed to dry \textit{in vacuo}, yielding a white solid, 0.45 g (71 \% yield).

\textit{M}p = 110-112 °C
$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.58 – 7.52 (m, 2H), 7.42 – 7.34 (m, 2H), 7.31 – 7.25 (m, 1H), 7.11 (s, 1H), 5.70 (d, $J = 88.1$ Hz, 2H), 3.54 (s, 2H), 2.44 (s, 3H).

$^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 173.02, 135.80, 133.95, 131.45, 128.89, 127.42, 125.42, 125.41, 125.40, 125.38, 124.86, 124.85, 35.88, 13.23.

FT-IR (solid sample): 3390, 3183, 1645, 1600, 1403 cm$^{-1}$

HRMS (ESI): calculated for C$_{13}$H$_{13}$NOS+1: 232.0796, found: 232.0975.

2.10.5.10 **Synthesis of 3-(1,2-dimethyl-1H-indol-3-yl)-4-(2-methyl-5-phenylthiophen-3-yl)-1H-pyrrole-2,5-dione**

Prepared based on literature procedure.$^{96}$ To a 25 mL RBF was added 2-(2-methyl-5-phenylthiophen-3-yl)acetamide (0.2 g, 8.65 x 10$^{-4}$ mol) and (0.23 g, 9.50 x 10$^{-4}$ mol), and THF (10 mL, anhydrous). The mixture was cooled to 0°C (ice/water bath) and treated drop wise over ~5 min with potassium tert-butoxide (1 M soln in THF, 2.6 mL 2.59 x 10$^{-3}$ mol). The solution was left to stir at 0 °C for 1 hr, at which point the bath was allowed to warm to RT and stir overnight. Next, 1 mL of HCl (concentrated) was poured into the RBF to neutralize the excess base. The flask contents were then extracted with EtOAc; the organic layer was washed with brine, dried over MgSO$_4$, filtered and concentrated under reduced pressure. The crude material was purified with column chromatography (silica gel, 25% EtOAc / hexanes), yielding a bright orange powder (0.19 g, 54%).

**M$p = 267-269$ °C (dec.)**
\( ^1H \) NMR (500 MHz, DMSO) \( \delta 11.08 \) (s, 1H), 7.59 – 7.51 (m, 2H), 7.47 – 7.36 (m, 4H), 7.33 – 7.27 (m, 1H), 7.13 – 7.04 (m, 2H), 6.92 – 6.86 (m, 1H), 3.71 (s, 3H), 2.21 (s, 3H), 1.75 (s, 3H).

\( ^{13}C \) NMR (126 MHz, DMSO) \( \delta 172.49, 172.23, 139.78, 139.55, 137.27, 135.16, 133.74, 131.16, 129.64, 129.59, 128.01, 125.85, 125.53, 125.44, 121.65, 121.64, 120.29, 119.74, 110.11, 102.88, 30.27, 15.00, 12.32. \)

HRMS (ESI): calculated for \( C_{25}H_{20}N_2O_2S: 413.1324 \), found: 413.1317.

UV-vis (EtOAc, 50 \( \mu \)M): \( \lambda_{\text{max}} = 442 \text{ nm}, \varepsilon = 6600 \text{ M}^{-1}\text{cm}^{-1}, \)

2.10.5.11 Synthesis of 3,4-dibromo-1-methyl-1H-pyrrole-2,5-dione

![Reaction Scheme]

Prepared based on literature procedure.\(^{109}\) To a 250 mL 3 neck RBF was added N-methylpyrrole (4 mL, 4.51 \( \times 10^{-2} \) mol), followed by ~100 mL of anhydrous THF. The resulting solution was cooled to ~65\(^\circ\)C (internal temp, dry ice/acetone) and then NBS (16.0 g 8.99 \( \times 10^{-2} \) mol) was added. The mixture was stirred at this temperature for 30 min and then the cooling bath was removed and the reaction contents were allowed to warm to room temperature gradually. The reaction was stirred for an additional 12 h at RT. Afterwards the solvent was removed and the remaining residue was suspended in hexanes and then filtered. The filtrate was collected and then concentrated under reduced pressure to yield a yellow oil which was immediately cooled to ~0\(^\circ\)C (ice / water bath) and 5 mL of nitric acid (70%) was added drop wise (exothermic!) over 45 min. After the addition was complete, the mixture was poured into 20 g of ice, once the ice melted, the mixture was extracted with ethyl acetate (3 \( \times \sim 100 \text{ mL} \)), the ethyl acetate layer was collected and washed with brine (\( \sim 100 \text{ mL} \)), followed by water (\( \sim 100 \text{ mL} \)), dried over MgSO\(_4\), filtered and concentrated to dryness. The crude residue was purified using
column chromatography, (silica gel, 15% EtOAc / hexanes), and afforded 5.9 g (49%) as pale yellow coloured flakes.

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 3.13 (s, 3H).

$^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 163.97, 129.39, 25.46.

2.10.5.12 Synthesis of 3-bromo-4-(2-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione

Prepared based on literature procedure.$^{93}$ To a RBF was added 25 mL of anhydrous THF and 2-methylindole (1.27 g, 9.68 x 10$^{-3}$ mol). The red coloured solution was treated with methylmagnesium bromide (3 M solution in diethyl ether, 3.3 mL). After the addition was complete, the solution was heated to 45 °C and kept at this temperature for 0.5 h. The solution was then allowed to cool to RT, and compound 3,4-dibromo-1-methyl-1H-pyrrole-2,5-dione (1.3 g, 4.83 x 10$^{-3}$ mol) was dissolved in 25 mL of anhydrous THF and added drop wise over ~0.5 hr via cannula into the flask containing indole. After the addition was complete, the reaction was heated to reflux and left to stir at this temperature for 2 hr. At which point it was cooled to RT, and 50 mL of cold citric acid (20% aq solution) was added and then extracted with ethyl acetate 2 x 100 mL. The EtOAc layers were combined and washed with water, dried over MgSO$_4$, filtered and concentrated to dryness under reduced pressure to afford a deep red brown solid. The material was pre-adsorbed on silica and purified using column chromatography (silica gel, 25% EtOAc / hexanes), yielding a vibrant red solid, 1.19 g (77%).

$^1$H NMR (400 MHz, DMSO-d$_6$) $\delta$ 11.76 (s, 1H), 7.39-7.36 (m, 2H), 7.14-7.09 (m, 1H), 7.08-6.99 (m, 1H), 3.02 (s, 3H), 2.42 (s, 3H).
\[ ^{13}\text{C} \text{ NMR} \ \delta \ (101 \text{ MHz, DMSO-d}_6) \ 168.77, 166.26, 139.08, 138.33, 135.62, 126.17, 121.35, 120.14, 111.09, 100.79, 24.58, 13.59. \]

**2.10.5.13 Synthesis of 3-bromo-2-methyl-5-phenylthiophene**

\[ \begin{align*}
\text{Br} & \quad \text{H}_3\text{C} \\
\text{S} & \quad \text{Br} \\
\text{Br} & \quad \text{H}_3\text{C} \\
\end{align*} \quad \xrightarrow{\text{Pd(PPh}_3)_4, \text{K}_2\text{CO}_3} \text{DME/H}_2\text{O, } \Delta \quad \text{Br} \\
\text{2.24} & \quad \xrightarrow{81\%} \quad \text{H}_3\text{C} \\
\end{align*} \]

Prepared based on literature procedure.\textsuperscript{100} To a 250 mL 3-neck RBF was added 50 mL of distilled water, 75 mL of dimethoxyethane, 2,4-dibromo-5-methylthiophene (3.03 g, 1.18 x 10\textsuperscript{-2} mol) and K\textsubscript{2}CO\textsubscript{3} (8.14 g, 5.89 x 10\textsuperscript{-2} mol). The yellow coloured solution was purged with N\textsubscript{2} for ~30 min. Phenylboronic acid (1.44 g, 1.18 x 10\textsuperscript{-2} mol) was added to the flask followed by Pd(PPh\textsubscript{3})\textsubscript{4} (0.21 g, 1.18 x 10\textsuperscript{-4} mol) and the reaction mixture was heated to reflux for 8 hr. The flask was allowed to cool to RT and the orange coloured solution was poured into 100 mL of water and extracted with DCM (100 mL x 2). The combined DCM layers were washed with brine (100 mL), water (100 mL), were dried over MgSO\textsubscript{4}, filtered and concentrated under reduced pressure to yield a white solid. The crude residue was purified with column chromatography (silica gel, 100% hexanes) to yield 2.43 g (81 %) of a white fluffy powder (with an intense fruit odour).

\[ ^1\text{H} \text{ NMR} \ (400 \text{ MHz, DMSO-d}_6) \ \delta \ 7.56-7.51 \ (m, \ 2H), 7.43-7.36 \ (m, \ 2H), 7.33-7.29 \ (m, \ 1H), 7.13 \ (s, \ 1H), 2.45 \ (s, \ 3H). \]
2.10.5.14 Synthesis of 1-methyl-3-(2-methyl-1H-indol-3-yl)-4-(2-methyl-5-phenylthiophen-3-yl)-1H-pyrrole-2,5-dione

To a 100 mL RBF was added 3-bromo-2-methyl-5-phenylthiophene (0.72 g, 2.86 x 10^{-3} mol) followed by anhydrous THF (50 mL). The clear and colourless solution was treated with n-BuLi (1.72 mL, 2 M hexanes) drop wise over 5 min at -60ºC, dry ice/acetone bath. The mixture was allowed to stir at this temperature for 20 min and then treated with tributylborate (0.93 mL, 3.44 x 10^{-3} mol). Stirred at this cooled temperature for 30 min before removing the cooling bath and allowing to warm to RT. Meanwhile, a solution of 60 mL DME and 40 mL of water, containing K$_2$CO$_3$ (2.02 g, 1.46 x 10^{-2} mol) and 3-bromo-4-(2-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (0.82 g, 2.58 x 10^{-3} mol) was purged with N$_2$ for 30 min. Pd(PPh$_3$)$_4$ (0.083 g, 7.2x10^{-5} mol) was added and then cannulated the now RT boronate ester (prepared earlier) into flask. The resulting suspension was heated to reflux overnight. Afterwards the heating source was removed and allowed to cool to RT. Water (100 mL) was poured into the flask contents and extracted with EtOAc (2 x 100 mL). The combined organic layers were washed with brine, followed by water, and then dried over MgSO$_4$, filtered and concentrated under reduced pressure. The deep red coloured residue was washed with DCM to yield a bright orange powder. The powder was recrystallized from hot ethanol, to yield small orange needles, 0.77 g (65%).

M$_p$ = 274-275 ºC

$^1$H NMR (500 MHz, DMSO) δ 11.58 (s, 1H), 7.54 (d, $J$ = 7.5 Hz, 2H), 7.42 (s, 1H), 7.40 (t, $J$ = 7.7 Hz, 2H), 7.34 – 7.25 (m, 2H), 7.06 (d, $J$ = 8.0 Hz, 1H), 7.01 (t, $J$ = 7.5 Hz, 1H), 6.83 (t, $J$ = 7.5 Hz, 1H), 3.04 (s, 3H), 2.17 (s, 3H), 1.75 (s, 3H)
\[^{13}\text{C} \text{NMR} \ (101 \ \text{MHz, DMSO}) \delta 171.35, 171.05, 139.89, 139.61, 138.69, 136.04, 134.83, 133.70, 129.96, 129.64, 129.60, 128.04, 126.80, 125.43, 125.39, 121.66, 120.00, 119.78, 111.35, 103.04, 24.49, 14.94, 13.53\]


UV-vis = (EtOAc, 50 \ \mu\text{M}): \(\lambda_{\text{max}} = 278 \ \text{nm}, \ \varepsilon = 26025 \ \text{M}^{-1}\text{cm}^{-1}; \lambda = 444 \ \text{nm}, \ \varepsilon = 5614 \ \text{M}^{-1}\text{cm}^{-1}\).

### 2.10.5.15 Synthesis of 3-(2-methyl-1H-indol-3-yl)-4-(2-methyl-5-phenylthiophen-3-yl)furan-2,5-dione

A flask was charged with 1-methyl-3-(2-methyl-1H-indol-3-yl)-4-(2-methyl-5-phenylthiophen-3-yl)-1H-pyrrole-2,5-dione (0.68 g, 1.65 \times 10^{-3} \ \text{mol}), ethanol (20 mL), and an aqueous KOH solution (10 mL of 20\% w/w). The solution was heated to reflux for 2 hr. At which point the reaction was cooled to RT, neutralized with aq HCl and the orange precipitate that formed was collected using vacuum filtration. The precipitate was washed thoroughly with water and then hexanes to yield a bright orange powder 0.59 g (90\% yield).

\(\text{Mp} = 262-263 \ ^\circ\text{C}\)

\[^{1}\text{H} \text{NMR} \ (400 \ \text{MHz, DMSO}) \delta 11.82 \ (s, 1\text{H}), 7.58 – 7.51 \ (m, 2\text{H}), 7.48 \ (s, 1\text{H}), 7.42 \ (t, \ J = 7.6 \ \text{Hz}, 2\text{H}), 7.35 – 7.28 \ (m, 2\text{H}), 7.12 – 7.02 \ (m, 2\text{H}), 6.88 \ (t, \ J = 7.6 \ \text{Hz}, 1\text{H}), 2.24 \ (s, 3\text{H}), 1.81 \ (s, 3\text{H}).\]

\[^{13}\text{C} \text{NMR} \ (101 \ \text{MHz, DMSO}) \delta 166.03, 165.87, 141.23, 140.49, 140.38, 136.64, 136.09, 133.45, 130.64, 129.71, 128.54, 128.25, 126.37, 125.50, 124.87, 122.14, 120.47, 119.89, 111.62, 102.52, 14.96, 13.61.\]
HRMS (ESI): calculated for C\textsubscript{24}H\textsubscript{17}NO\textsubscript{3}S\textsuperscript{+1}: 400.100291, found: 400.100311

UV-vis = (THF, 25 µM): $\lambda_{\text{max}} = 283$ nm, $\varepsilon = 18230$ M\textsuperscript{-1}cm\textsuperscript{-1}; $\lambda = 440$ nm, $\varepsilon = 5389$ M\textsuperscript{-1}cm\textsuperscript{-1}.

2.10.5.16 Synthesis of 3-(2-methyl-1H-indol-3-yl)-4-(2-methyl-5-phenylthiophen-3-yl)-1H-pyrrole-2,5-dione

Anhydride 3-(2-methyl-1H-indol-3-yl)-4-(2-methyl-5-phenylthiophen-3-yl)furan-2,5-dione (0.79 g, 1.98 x 10\textsuperscript{-3} mol) was dissolved in DMF (12 mL). Next an excess of ammonium acetate (4.11 g, 5.33 x 10\textsuperscript{-2} mol) was added and the resulting mixture heated to ~100°C overnight. The flask contents were allowed to cool to room temperature and then extracted with EtOAc (2 x 50 mL). The EtOAc layers were combined and then washed with brine (50 mL), followed by water (2 x 50 mL), dried over MgSO\textsubscript{4}, filtered and concentrated under reduced pressure to yield a bright orange solid. The solid was washed with DCM and Et\textsubscript{2}O the yield desired product, 0.66 g (84%).

Mp = 250-255 °C

\textsuperscript{1}H NMR (400 MHz, DMSO) $\delta$ 11.55 (s, 1H), 11.06 (s, 1H), 7.55 (d, $J = 7.5$ Hz, 2H), 7.47 – 7.35 (m, 3H), 7.35 – 7.24 (m, 2H), 7.12 – 6.96 (m, 2H), 6.84 (t, $J = 7.5$ Hz, 1H), 2.18 (s, 3H), 1.75 (s, 3H).

\textsuperscript{13}C NMR (101 MHz, DMSO-d\textsubscript{6}) $\delta$ 172.01, 171.73, 139.28, 139.01, 138.02, 135.53, 134.91, 133.25, 130.23, 129.18, 129.14, 127.51, 126.35, 125.03, 124.94, 121.11, 119.47, 119.25, 110.81, 102.54, 14.42, 13.03.
HRMS (ESI): calculated for C_{24}H_{18}N_{2}O_{2}S+1: 399.1161 g/mol, found: 399.1156 g/mol

UV-vis (THF, 50 μM): \( \lambda_{\text{max}} = 278 \text{ nm}, \varepsilon = 21810 \text{ M}^{-1}\text{cm}^{-1}; \lambda = 445 \text{ nm}, \varepsilon = 5800 \text{ M}^{-1}\text{cm}^{-1} \)

2.10.5.17 *Synthesis of 3-bromo-5-(4-methoxyphenyl)-2-methylthiophene*

Prepared based on literature procedure.\(^{118}\) A mixture of 3,5-dibromo-2-methylthiophene (0.51 g, 2.00 \( \times \) 10\(^{-3} \) mol), \( \text{K}_2\text{CO}_3 \) (1.35 g, 9.77 \( \times \) 10\(^{-3} \) mol), \( \rho \)-methoxyboronic acid (0.33 g, 2.15 \( \times \) 10\(^{-3} \) mol), and \( \text{Pd(PPh}_3)_4 \) (0.11 g, 9.52 \( \times \) 10\(^{-5} \) mol) in 25 mL DME/H\(_2\)O (1.5:1) was heated to reflux under nitrogen for 16 h. Afterwards the mixture was cooled to RT, poured into water (50 mL) and extracted with DCM (2 \( \times \) 50 mL). The combined organic layers were washed with brine (50 mL) and water (50 mL), then dried over MgSO\(_4\), filtered and concentrated under reduced pressure. The resulting crude material was purified using column chromatography (silica gel, 100% hexanes), yielding an off-white powder (0.41 g, 73%).

\(^1\text{H NMR (400 MHz, CDCl}_3\) δ 7.46 (d, \( J = 8.9 \text{ Hz}, 2\text{H} \)), 7.01 (s, 1\text{H} \)), 6.92 (d, \( J = 8.9 \text{ Hz}, 2\text{H} \)), 3.85 (s, 3\text{H} \)), 2.43 (s, 3\text{H} \)).
**Synthesis of 3-(5-(4-methoxyphenyl)-2-methylthiophen-3-yl)-1-methyl-4-(2-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione**

To a 25 mL pear shaped flask containing 3-bromo-5-(4-methoxyphenyl)-2-methylthiophene (0.26 g, 9.14 x 10^{-4} moles) was added ~15 mL of THF (anhydrous, SPS) and the clear and colourless solution was cooled to ~ -70°C using a dry ice acetone bath. Added n-BuLi (0.4 mL, 1.01x10^{-3} moles) drop wise and upon addition completion, allowed the reaction to stir at this cooled temperature for an additional 30 minutes before adding tributylborate (0.27 mL, 1.01 x 10^{-3} moles) and let the reaction mixture warm slowly to RT over ~1 hr. A three neck RBF was charged with 3-bromo-4-(2-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (0.26 g, 8.21 x 10^{-4} mol), K_{2}CO_{3} (0.60 g, 4.34 x 10^{-3} mol), and 40 mL THF/H_{2}O (1:1), and purged with N_{2} for 30 minutes. Pd(PPh\textsubscript{3})\textsubscript{4} (0.05 g, 4.56 x 10^{-5} mol) was added and then the cooled boronate ester prepared above was cannulated into the reaction flask and heated to reflux for ~15 h. The resulting deep orange/red solution was then cooled to RT, poured into a separatory funnel containing 50 mL of H_{2}O. The mixture was extracted with EtOAc (50 mL x 2) and the organic layers were collected, washed with brine, water, and dried over MgSO_{4}, filtered and concentrated under reduced pressure. The deep red oil isolated after evaporation was purified using column chromatography (silica gel, using a gradient eluent system varying the concentration from 25% EtOAc / hexanes to 40% EtOAc / hexanes). Orange solid 0.124 g, (31%).

\textsuperscript{1}H NMR (400 MHz, DMSO) δ 11.57 (s, 1H), 7.50 – 7.43 (m, 2H), 7.33 – 7.26 (m, 2H), 7.09 – 6.95 (m, 4H), 6.88 – 6.81 (m, 1H), 3.78 (s, 3H), 3.05 (s, 3H), 2.17 (s, 3H), 1.74 (s, 3H).
\[ ^{13}\text{C} \text{NMR (101 MHz, DMSO) } \delta 171.36, 171.06, 159.32, 139.90, 138.64, 138.45, 136.02, 134.69, 130.11, 129.43, 126.82, 126.44, 124.09, 121.64, 119.98, 119.80, 115.04, 111.32, 111.30, 103.06, 55.71, 24.48, 14.88, 13.52. \]

HRMS (ESI): calculated for \( 443.1424 \), \( \text{C}_{26}\text{H}_{23}\text{N}_{2}\text{O}_{2}\text{S} (\text{M}+\text{H}) \), found: 443.1417

UV-vis (THF, 50 \( \mu \text{M} \)): \( \lambda_{\text{max}} = 284 \text{ nm} \), \( \varepsilon = 20140 \text{ M}^{-1}\text{cm}^{-1} \); \( \lambda = 434 \text{ nm} \), \( \varepsilon = 5937 \text{ M}^{-1}\text{cm}^{-1} \)

2.10.5.19 Synthesis of 3-(5-(4-methoxyphenyl)-2-methylthiophen-3-yl)-4-(2-methyl-1H-indol-3-yl)-1H-pyrrrole-2,5-dione

Dissolved 3-(5-(4-methoxyphenyl)-2-methylthiophen-3-yl)-1-methyl-4-(2-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (0.05 g, \( 1.11 \times 10^{-4} \text{ mol} \)) in EtOH in a 25 mL RBF, added aq. KOH soln (20% KOH) to the RBF attached a reflux condenser and heated to reflux. Turned to a yellow/clear colour (was originally orange and cloudy). After 3 hr, cooled to the reaction mixture to RT, neutralized with 2N HCl, and collected the orange precipitate using centrifugation. The supematant was removed the pellet washed several times with water. The crude product used directly in the next step without further purification.

Dissolved 3-(5-(4-methoxyphenyl)-2-methylthiophen-3-yl)-4-(2-methyl-1H-indol-3-yl)furan-2,5-dione (isolated from the previous step) in 10 mL of DMF. Transferred the resulting bright orange solution to a 1 neck 25 mL RBF and added ammonium acetate (5.44 g, 7.06 \( \times 10^{-2} \text{ mol} \)) in one portion. The solution was then heated to \( \sim 120^\circ\text{C} \) for 6 h. At which point it was cooled to RT, the contents of the reaction flask were poured into a separatory funnel containing 10 mL of water, and was extracted with EtOAc (2 x 10 mL). The organic layers were combined and washed with water several times, then dried over MgSO\(_4\), filtered and concentrated under reduced pressure to yield a bright orange solid (0.29 g, 60% yield for two steps).
$^1$H NMR (400 MHz, DMSO) δ 11.54 (s, 1H), 11.04 (s, 1H), 7.51 – 7.42 (m, 2H), 7.32 – 7.25 (m, 2H), 7.08 – 6.93 (m, 4H), 6.88 – 6.80 (m, 1H), 3.78 (s, 3H), 2.17 (s, 3H), 1.74 (s, 3H).

$^{13}$C NMR (101 MHz, DMSO) δ 172.52, 172.24, 159.30, 139.79, 138.47, 138.34, 136.00, 135.26, 130.86, 129.51, 126.86, 126.81, 126.48, 124.20, 121.57, 119.93, 119.75, 115.02, 109.98, 103.05, 55.70, 14.84, 13.51.

HRMS (ESI): calculated for 429.1267, C$_{25}$H$_{21}$N$_2$O$_3$S (M+H), found: 429.1259.

UV-vis (hexanes/THF (9:1), 65 µM): $\lambda_{\text{max}} = 278$ nm, $\varepsilon = 25077$ M$^{-1}$cm$^{-1}$; $\lambda = 435$ nm, $\varepsilon = 6740$ M$^{-1}$cm$^{-1}$.

2.10.5.20 Synthesis of 4-(4-bromo-5-methylthiophen-2-yl)-N,N-dimethylaniline

Prepared based on literature procedure.$^{119}$ To a 3 neck RBF was added DME (75 mL) and H$_2$O (25 mL), followed by K$_2$CO$_3$ (7.83 g, 5.67 x 10$^{-2}$ mol). The solution was purged with N$_2$ for 30 min and then 2,4-dibromo-5-methylthiophene (2.89 g, 1.13 x 10$^{-2}$ mol), the 4-(dimethylamino)phenyl boronic acid (1.87 g, 1.13 x 10$^{-2}$ mol) and Pd(PPh$_3$)$_4$ (0.10 g, 8.83 x 10$^{-5}$ mol) were added and the reaction mixture was heated to reflux overnight. At which point the reaction mixture was cooled to RT, poured into 100 mL water, and extracted with DCM (2 x 100 mL). The organic layers were combined and washed with brine (50 mL) and water (50 mL), followed by drying over MgSO$_4$, filtration and concentration under reduced pressure. The crude product was purified using column chromatography (silica gel, 10% EtOAC / hexanes) to give a white solid (2.27 g, 68% yield).

$^1$H NMR (500 MHz, CDCl$_3$) δ 7.39 (d, $J = 8.6$ Hz, 2H), 6.94 (s, 1H), 6.73 (m, 2H), 2.99 (s, 6H), 2.39 (s, 3H).
2.10.5.21 Synthesis of 3-(5-(dimethylamino)phenyl)-2-methylthiophen-3-yl)-1-methyl-4-(2-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione

To a 25 mL pear shaped flask containing 4-(4-bromo-5-methylthiophen-2-yl)-N,N-dimethylaniline (0.34 g, 1.15 x 10⁻³ mol) was added ~15 mL of THF (anhydrous, SPS) and solution was cooled to ~ -60°C using a dry ice acetone bath. Next, n-BuLi (0.5 mL, 1.26 x 10⁻³ mol, 2.5 M solution in hexanes) was added to the reaction flask drop wise and upon addition completion, allowed the reaction to stir at this cooled temperature for an additional 30 min. Tributylborate (0.34 mL, 1.26 x 10⁻³ moles) was then added and the cooling bath was removed and the reaction mixture was allowed to warm slowly to RT over ~1 hr. A separate three neck RBF was charged with 3-bromo-4-(2-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (0.33 g, 1.03 x 10⁻³ mol), K₂CO₃ (0.79 g, 5.73 x 10⁻³ mol), and 50 mL DME/H₂O (1:1), and purged with N₂ for 30 minutes. Pd(PPh₃)₄ (0.06 g, 5.19 x 10⁻⁵ mol) was added and then the cooled boronate ester prepared above was cannulated into the reaction flask and heated to reflux overnight. The mixture was then cooled to RT and then water was added the flask. The mixture was extracted with EtOAc (50 mL x 2) and the organic layers were collected, washed with brine, water, and dried over MgSO₄, filtered and concentrated under reduced pressure. The brown residue isolated after drying was washed with DCM and hexanes to yield the pure product as a deep maroon coloured microcrystalline solid 0.16 g, (36%).

mp = >252 °C.

¹H NMR (500 MHz, DMSO) δ 11.56 (s, 1H), 7.35 (d, J = 8.8 Hz, 2H), 7.30 (d, J = 8.0 Hz, 1H), 7.17 (s, 1H), 7.08 (d, J = 7.9 Hz, 1H), 7.02 (t, J = 7.7 Hz, 1H), 6.84 (t, J =
7.5 Hz, 1H), 6.74 (d, J = 8.9 Hz, 2H), 3.05 (s, 3H), 2.93 (s, 6H), 2.17 (s, 3H), 1.72 (s, 3H).

$^{13}$C NMR (101 MHz, DMSO) δ 171.40, 171.10, 150.29, 140.95, 138.59, 137.18, 136.01, 134.48, 130.31, 129.29, 126.86, 126.34, 122.46, 121.74, 121.61, 119.95, 119.83, 112.97, 111.30, 109.98, 103.11, 24.47, 14.87, 13.52.

UV-vis (hexanes/THF (95:5), 50 µM): $\lambda_{\text{max}}$ = 285 nm, $\varepsilon$ = 17165 M⁻¹cm⁻¹; $\lambda$ = 325, $\varepsilon$ = 19880 M⁻¹cm⁻¹; $\lambda$ = 446 nm, $\varepsilon$ = 6218 M⁻¹cm⁻¹.

HRMS (ESI): calculated for C$_{27}$H$_{25}$N$_3$O$_2$S+1: 456.1746, found: 456.1748.

2.10.5.22 Synthesis of 3-(2,5-dimethylthiophen-3-yl)-1-methyl-4-(2-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione

To a 25 mL pear shaped flask containing 3-bromo-2,5-dimethylthiophene (0.80 g, 4.19 x 10⁻³ mol) was added ~20 mL of THF (anhydrous, SPS) and the clear and colourless solution was cooled to ~ -70ºC using a dry ice acetone bath. Next, n-BuLi (1.84 mL, 4.61x10⁻³ mol) was added drop wise and allowed to stir at this cooled temperature for an additional 30 minutes before adding tributylborate (1.24 mL, 4.61 x 10⁻³ mol). The cooling bath was removed shortly after the borate addition and the reaction mixture was allowed to warm slowly to RT over ~1 hr. A separate three neck RBF was charged with 3-bromo-4-(2-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (0.67 g, 2.10 x 10⁻³ mol), K$_2$CO$_3$ (2.31 g, 1.67 x 10⁻² mol), 75 mL THF/H$_2$O (1:1), and purged with N$_2$ for 30 minutes. Pd(PPh$_3$)$_4$ (0.14 g, 1.05 x 10⁻⁴ mol) and the cooled boronate ester prepared above was cannulated into the reaction flask and heated to reflux for ~15 h. The resulting deep orange/red solution was then cooled to RT, poured into a separatory funnel containing 50 mL of H$_2$O. The mixture was extracted with EtOAc (100 mL x 2) and
the organic layers were collected, washed with brine, water, and dried over MgSO₄, filtered and concentrated under reduced pressure. The red orange oil isolated after evaporation was purified using column chromatography (silica gel, 25% EtOAc / hexanes) to give a bright orange solid 0.94 g, 64% yield.

Mp = 226-227 °C (dec.)

¹H NMR (500 MHz, DMSO) δ 11.53 (s, 1H), 7.29 (d, J = 8.0 Hz, 1H), 7.07 – 6.98 (m, 2H), 6.86 (t, J = 7.5 Hz, 1H), 6.74 (s, 1H), 3.02 (s, 3H), 2.37 (s, 3H), 2.11 (s, 3H), 1.66 (s, 3H).

¹³C NMR (400 MHz, DMSO) = ¹³C NMR (101 MHz, DMSO) δ 171.41, 171.13, 138.43, 137.66, 135.98, 135.91, 134.20, 130.48, 128.00, 127.22, 126.88, 121.56, 119.91, 119.80, 111.27, 103.12, 24.44, 15.27, 14.73, 13.42.

HRMS (ESI): calculated for 351.11618, C₂₀H₁₉N₂O₂S (M+H), found: 351.11633

UV-visible absorption spectrum (THF, 50 µM): λ max = 445 nm, ε = 6018 M⁻¹cm⁻¹

2.10.5.23 Synthesis of 3,4-bis(2-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione

Prepared based on literature procedure.³³ In a three-neck RBF equipped with condenser, a mixture of 2-methylindeole (2.31 g, 1.76 x 10⁻² mol in toluene (75 mL, anhydrous distilled from CaH₂) was treated drop wise with methyl magnesium bromide (5.9 mL, 1.76 x 10⁻² mol, 3M solution Et₂O) over ~ 2 min. Solution turned from orange/red and clear to deep purple/black upon addition. The mixture was heated to
~45°C for 30 min then allowed to cool to RT. A suspension of 3,4-dibromomaleimide (1.08 g, 4.24 x 10⁻³ mol) in 75 mL toluene was cannulated into the reaction mixture over ~1 h at RT. Once finished the reaction was heated to reflux for 1 hr. Cooled the flask to RT, then poured cold 20% citric acid into the RBF. Next poured the flask contents into a separatory funnel, added 250 mL of EtOAc. Separated the layer, and extracted the aqueous layer with EtOAc (2 x 100 mL). Washed the combined organic layers with brine (250 mL) and then water (250 mL). Dried over MgSO₄, filtered and concentrated on under reduced pressure to yield the crude material as a red oil. This was purified using column chromatography, (silica gel 10% EtOAc / hexanes to 50% EtOAc / hexanes). The purified material isolated as a deep red coloured powder, 0.40 g (39%).

Mp = >230 °C

¹H NMR (400 MHz, DMSO) δ 11.27 (s, 2H), 10.85 (s, 1H), 7.22 (d, J = 8.0 Hz, 2H), 7.01 (d, J = 7.8 Hz, 2H), 6.98 – 6.92 (m, 2H), 6.75 (t, J = 7.3 Hz, 2H), 1.97 (s, 6H).

¹³C NMR (101 MHz, DMSO) δ 172.88, 135.90, 132.36, 127.11, 121.19, 119.76, 119.56, 111.05, 103.74, 13.45.

UV-vis (THF, 50 µM): λ_max = 278 nm, ε = 13910 M⁻¹cm⁻¹; λ = 450 nm, ε = 6221 M⁻¹cm⁻¹.

2.10.5.24 Synthesis of 3,4-bis(2-methyl-1H-indol-3-yl)-1H-pyrrol-2(5H)-one

Prepared based on literature procedure.⁶⁸a A flask was charged with 3,4-bis(2-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (0.39 g, 1.10 x 10⁻³ mol), acetic acid (30 mL, glacial) and tin powder (3.40 g, 2.86 x 10⁻² mol). The suspension was heated to ~100°C and treated drop wise HCl (concentrated, 9 mL) over 1 h. The suspension turned from red/orange to a pale yellow/clear. Let stir for an additional 1 hr before cooling to RT.
Afterwards, the solution was filtered through celite, and the celite was washed with THF (~10 mL) and acetone (~10 mL). The combined washings were cooled and the resulting precipitate was washed collected by centrifugation. The pellet was resuspended in water and centrifuged. The pellet was collected and then dried in vacuo, to yield a white powder (0.12 g, 32 % yield).

\[ ^1H \text{ NMR (400 MHz, DMSO)} \delta 11.05 (s, 1H), 10.90 (s, 1H), 8.22 (s, 1H), 7.42 (d, J = 7.8 Hz, 1H), 7.20 (dd, J = 11.2, 8.0 Hz, 2H), 7.05 – 6.83 (m, 4H), 6.71 – 6.63 (m, 1H), 4.46 (d, J = 30.1 Hz, 2H), 2.03 (s, 3H), 1.77 (s, 3H). \]

2.10.5.25  
**Synthesis of 5-methylthiophene-2-carbaldehyde**

\[ \text{H}_3\text{C} \overset{\text{S}}{\text{S}} \text{H}_3 \overset{\text{C}}{\overset{\text{H}}{\text{O}}} \text{1) n-BuLi, THF, -60°C} \]
\[ \text{2) DMF -60°C to RT} \]
\[ \text{3) HCl (aq)} \]
\[ 94\% \]

To a 100 mL RBF was added 2-methylthiophene (6.98 g, 7.11 x 10^-2 mol) followed by THF (~100 mL, anhydrous). The clear and colourless solution was cooled to ~ -60°C (dry ice / acetone) and n-BuLi (37.0 mL, 7.47 x 10^-2 mol, 2 M soln in hexanes) was added drop wise over 30 minutes and left to stir at this cooled temperature for an additional 30 min. DMF (8.3 mL, 1.07 x 10^-1 mol) was then added in one portion, and after stirring for 30 min at this cooled temperature, the cooling bath was removed and the reaction flask was allowed to come to room temperature. Afterwards, the reaction mixture was treated with 100 mL of 2 M HCl. This was followed by extraction with Et_2O (2 x100 mL). The combined organic layers were washed with brine, NaHCO_3, water (~50 mL of each). The organic layer was collected, dried over MgSO_4, filtered and concentrated under reduced pressure to yield a viscous yellow oil. The crude material was purified using column chromatography, silica gel, 10% EtOAc / hexanes (8.42 g, yield 94%).

\[ ^1H \text{ NMR (500 MHz, CDCl}_3\text{)} \delta 9.83 (s, 1H), 7.62 (d, J = 3.7 Hz, 1H), 6.91 (dd, J = 3.6, 0.7 Hz, 1H), 2.60 (s, 4H). \]
2.10.5.26  Synthesis of (E)-3-(4-bromo-5-methylthiophen-2-yl)acrylic acid

![Synthesis of (E)-3-(4-bromo-5-methylthiophen-2-yl)acrylic acid](image)

Prepared based on literature procedure. Added 4-bromo-5-methylthiophene-2-carboxaldehyde (5.30 g, 2.58 x 10^-2 mol) to a 1 neck 100 mL RBF, followed by 40 mL of pyridine and malonic acid (10.77 g, 1.03 x 10^-1 moles) and stirred briefly until the malonic acid was dissolved. After piperidine 2.4 mL was added, and a reflux condenser was attached. The mixture was heated to 80°C for 2 hr and then refluxed for an additional 1 hr. Afterwards the solution was cooled to RT and poured into an aqueous solution of HCl (aq). The resulting precipitate that formed was collected using vacuum filtration, yielding an off-white powder, this was washed thoroughly with water (4.81 g, 75% yield)

^1H NMR (400 MHz, DMSO) δ 12.44 (s, 1H), 7.62 (d, J = 15.7 Hz, 1H), 7.46 (s, 1H), 6.13 (d, J = 15.8 Hz, 1H), 2.38 (s, 3H).

^13C NMR (101 MHz, DMSO) δ 167.50, 138.00, 136.79, 136.13, 133.69, 118.23, 110.35, 15.33.

2.10.5.27  Synthesis of 3-(4-bromo-5-methylthiophen-2-yl)propan-1-ol

![Synthesis of 3-(4-bromo-5-methylthiophen-2-yl)propan-1-ol](image)

To a 250 mL 3-neck RBF was added THF (100 mL, anhydrous), followed by LiAlH₄ (1.11 g, 2.92 x 10^-2 mol). To the stirred suspension was added a 25 mL solution of 3-(4-bromo-5-methylthiophen-2-yl)acrylic acid (3.56 g, 1.44 x 10^-2 mol) in THF drop wise via cannula, (exothermic) added over 10 minutes. Afterwards heated to reflux for ~3 hr. Next the flask contents were cooled to RT, poured into a separatory funnel containing
~100 mL of water, and then added 50 mL of 10% H₂SO₄. The mixture was extracted with 
Et₂O, washed with brine, NaHCO₃ and water. The organic layer was dried over MgSO₄,  
filtered and concentrated under reduced pressure to yield a yellow oil. The crude  
material was purified using silica gel, 30% EtOAc / hexanes, to yield a colourless, viscous oil (1.85 g, 55% yield)  

\[ \text{H NMR (400 MHz, DMSO-d₆)} \delta 6.74 \text{ (s, 1H), 4.52 (t, J = 5.1 Hz, 1H), 3.46 – 3.38 (m, 2H), 2.75 (t, J = 7.6 Hz, 2H), 2.30 (s, 3H), 1.78 – 1.65 (m, 2H).} \]

\[ \text{C NMR (101 MHz, DMSO) \delta 143.15, 131.50, 126.98, 107.86, 60.03, 34.49, 26.31, 14.69.} \]

2.10.5.28 Synthesis of 3-(4-bromo-5-methylthiophen-2-yl)propyl 4-methylbenzenesulfonate

A flask was charged with 3-(4-bromo-5-methylthiophen-2-yl)propan-1-ol (1.62 g, 6.89 x 10⁻³ mol), DCM (25 mL), p-toluenesulfonyl chloride (1.66 g, 8.71 x 10⁻³ mol) and triethylamine (1.67 mL, 1.20 x 10⁻² mol). The resulting bright yellow coloured solution was left to stir at RT overnight. Then the flask contents were poured into a separatory funnel containing water, extracted with DCM (2 x 50 mL). The combined the DCM layers were washed with brine (sat’d), dried over MgSO₄, filtered and concentrated under reduced pressure. The crude residue was passed through a plug of silica gel (eluted with 10% EtOAc / hexanes), yielding a pale yellow oil (2.0 g, ~90%).

\[ \text{H NMR (400 MHz, CDCl₃)} \delta 7.88 – 7.74 \text{ (m, 2H), 7.44 – 7.33 (m, 2H), 6.43 (s, 1H), 4.07 (t, J = 6.0 Hz, 2H), 2.78 (t, J = 7.3 Hz, 2H), 2.49 (s, 3H), 2.33 (s, 3H), 2.02 – 1.92 (m, 2H).} \]
2.10.5.29  Synthesis of 3-(4-bromo-5-methylthiophen-2-yl)-N,N-dimethylpropan-1-amine

A sealable Schlenk tube was charged with 3-(4-bromo-5-methylthiophen-2-yl)propyl4-methylbenzenesulfonate (1.49 g, 3.83 x 10^{-3} mol) and then dissolved in benzene (10 mL). A solution of dimethylamine (9.5 mL, 1.91 x 10^{-2} mol, 2 M solution in THF) was added in one portion and the flask was sealed and heated overnight in a 70 °C oil bath. Afterwards the flask was removed from the bath and allowed to cool to RT, poured the contents of the flask into a separatory funnel and added EtOAc (~50 mL) followed by water (~50 mL). The layers were separated and the water layer was extracted (2 x 50 mL) with EtOAc. The combined organic layers were washed with brine and water, dried over MgSO \(_4\), filtered and concentrated under reduced. The brownish/yellow oil obtained was purified the crude residue using column chromatography silica gel, 5% MeOH/DCM to a white solid, 0.85 g, 71%.

\(^{1}\)H NMR (400 MHz, DMSO) \(\delta 6.74\) (s, 1H), 2.72 (t, \(J = 7.5\) Hz, 2H), 2.30 (s, 3H), 2.21 (t, \(J = 7.1\) Hz, 2H), 2.11 (s, 6H), 1.68 (quintet, \(J = 7.2\) Hz, 2H).

\(^{13}\)C NMR (101 MHz, DMSO) \(\delta 143.09, 131.56, 127.03, 107.87, 58.38, 45.61, 29.21, 27.39, 14.70\).

2.10.5.30  Synthesis of 3-(5-(3-(dimethylamino)propyl)-2-methylthiophen-3-yl)-4-(2-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione

1) n-BuLi, THF, -60°C
2) tributyl borate -60°C to RT
3) 2.44, Pd(PPh\(_3\))\(_4\), K\(_2\)CO\(_3\)

\(\text{DME/H}_2\text{O, } \Delta\) 38%
To a 50 mL pear shaped flask was added 3-(4-bromo-5-methylthiophen-2-yl)-
N,N-dimethylpropan-1-amine (0.70 g, 2.67 x 10^{-3} mol) followed by THF (20 mL, anhydrous). The solution was cooled to -60 °C (acetone / dry ice) and then treated with n-BuLi drop wise (1.1 mL, 2.82 x 10^{-3} mol, 2.5 M solution in hexanes). The solution changed from orange/clear to yellow/clear. Let stir at this cooled temperature for an additional 15 min, afterwards quenched the reaction mixture with tributylborate (1.1 mL, 4.03 x 10^{-3} mol), let stir for 30 min before removing the cooling bath and letting the solution warm to RT. For the second step of the reaction, a flask was charged with water (20 mL) and DME (15 mL), followed by K$_2$CO$_3$ (1.85 g, 1.34 x 10^{-2} mol) and 3-bromo-4-
(2-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (0.68 g, 2.24 x 10^{-3} mol). The resulting deep orange/red solution was purged with nitrogen for 30 min. Afterwards, the Pd(PPh$_3$)$_4$
(0.068 g, 5.88 x 10^{-5} mol) was added, followed by the RT tributylborate mixture (via cannula) and then the resulting suspension was heated to reflux for 4 hr. The reaction was monitored by TLC, and after consumption of the starting material, the flask was removed from the heat source and allowed to cool to RT. The flask contents were poured into water and extracted with EtOAc (2 x 100 mL). The combined organic layers were washed with brine (2 x 50 mL) and water (2 x 50 mL), then dried over MgSO$_4$, filtered and concentrated under reduced pressure, giving an orange oil. The crude material was purified using column chromatography, silica gel, gradient 2-5% MeOH/DCM, yielding an orange powder (0.41 g, 38% yield).

Mp = 140-145 °C

$^1$H NMR (400 MHz, DMSO) $\delta$ 11.50 (s, 1H), 10.96 (s, 1H), 7.28 (d, $J = 8.0$ Hz, 1H), 7.05 – 6.95 (m, 2H), 6.82 (t, $J = 7.5$ Hz, 1H), 6.71 (s, 1H), 2.70 (t, $J = 7.1$ Hz, 2H), 2.21 – 2.06 (m, 11H), 1.69 (d, $J = 6.9$ Hz, 3H), 1.68 – 1.59 (m, 2H).

$^{13}$C NMR (101 MHz, DMSO) $\delta$ 172.53, 172.30, 141.27, 138.33, 137.41, 135.99, 134.90, 131.24, 127.92, 126.87, 126.53, 121.48, 119.79, 111.22, 109.99, 103.04, 58.02, 45.53, 29.33, 27.06, 14.76, 13.43.

HRMS (ESI): calculated for C$_{23}$H$_{26}$N$_3$O$_2$S 408.17403 (M+H), found: 408.17586.

UV-visible (50 µM, THF): $\lambda_{max} = 435$ nm, $\varepsilon = 4945.6$ M$^{-1}$ cm$^{-1}$
2.10.5.31 Synthesis of the ring-closed isomer of 3-(5-(3-(dimethylamino)propyl)-2-methylthiophen-3-yl)-4-(2-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione.

A solution of 3-(5-(3-(dimethylamino)propyl)-2-methylthiophen-3-yl)-4-(2-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (9.7 mg, $2.4 \times 10^{-3}$ moles/L) in anhydrous THF (10 mL) was prepared and transferred to a scintillation vial containing a stir bar. The vial was irradiated with 440 nm light (fluorimeter source, slit width 10 nm) while stirring for a total of 90 minutes. The THF was removed under reduced pressure (all manipulations performed in the dark), the deep red/purple residue was re-dissolved in DCM / 2.5% MeOH and purified using column chromatography (silica gel, DCM / 2.5% MeOH / 2.5% TEA), affording 3.6 mg of the ring-closed isomer (37% yield).

$^1$H NMR (400 MHz, DMSO) $\delta$ 11.07 (s, 1H), 8.38 – 8.28 (m, 1H), 7.31 – 7.15 (m, 1H), 6.80 – 6.64 (m, 3H), 6.59 (s, 1H), 2.62 (t, $J = 7.1$ Hz, 2H), 2.38 – 2.28 (m, 2H), 2.19 (s, 6H), 1.78 – 1.67 (m, 2H), 1.65 (s, 3H), 1.58 (s, 3H).

UV-vis (THF, 14 µM): $\lambda = 318$ nm ($\varepsilon = 7348$ cm$^{-1}$M$^{-1}$), 388 nm ($\varepsilon = 10288$ cm$^{-1}$M$^{-1}$), 529 nm ($\varepsilon = 5384$ cm$^{-1}$M$^{-1}$).

2.10.5.32 Synthesis of 2,3-dibromomaleic anhydride

Prepared based on literature procedure.$^{114}$ Maleic anhydride (0.98 g, $1.00 \times 10^{-2}$ mol) was added to a 100 mL Kontes$^\text{TM}$ Schlenk tube, followed by AlCl$_3$ (0.04 g, $3.00 \times 10^{-4}$ mol) and bromine (1.00 mL, $2.01 \times 10^{-2}$ mol). The tube was briefly flushed with
nitrogen, sealed and placed into a temperature-controlled oil bath. The mixture was gradually warmed to 130°C and then kept at this temperature for an additional 14 h, yielding pale yellow needles. The tube was removed from the oil bath, allowed to cool to room temperature and then opened cautiously allowing the vapours to escape. The mixture was dissolved in EtOAc (~25 mL) and filtered. The filtrate was collected and the solvent was removed under reduced pressure to afford 2.39 g (93%) of the product as white shiny flakes.

$^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 158.52, 131.35.

2.10.5.33 Synthesis of 2,3-dibromomaleimide.

![Synthesis of 2,3-dibromomaleimide](image)

A mixture of 2,3-dibromomaleic anhydride (6.50 g, $2.54 \times 10^{-2}$ mol), ammonium acetate (3.14 g, $4.07 \times 10^{-2}$ mol) and acetic acid (25 mL) was heated at reflux for 4 h. The reaction was allowed to cool to room temperature and the solvent was removed under reduced pressure. The pale brown residue was suspended in water (25 mL) and filtered yielding 3.66 g (57%) of the product as an off-white solid.

$^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 11.68 (s, 1H).

2.10.5.34 Synthesis of 3-bromo-4-(2-methyl-1H-indol-3yl)-1H-pyrrole-2,5-dione.

![Synthesis of 3-bromo-4-(2-methyl-1H-indol-3yl)-1H-pyrrole-2,5-dione](image)
Prepared based on literature procedure. A solution of 2-methylindole (3.56 g, 2.71 × 10⁻² mol) in anhydrous THF (75 mL) was treated drop wise over 5 min with methylmagnesium bromide (3.0 M solution in Et₂O, 9.0 mL, 2.71 × 10⁻² mol) at room temperature. After the addition was complete, the solution was heated to 45°C and kept at this temperature for 30 min. The reaction was allowed to cool to room temperature at which time it was treated with a solution of 2,3-dibromomaleimide (3.32 g, 1.30 × 10⁻² moles) in anhydrous THF (25 mL) drop wise over 30 min using a cannula. After the addition was complete, the reaction mixture was heated at reflux for 2 h. The reaction was allowed to cool to room temperature, treated with cold citric acid (100 mL of a 20% solution in water) and extracted with EtOAc (2 × 100 mL). The organic layers were combined, washed with water then a saturated sodium chloride solution, dried over MgSO₄, filtered and concentrated under reduced pressure to afford a dark red solid. The residue was pre-adsorbed on silica and purified using column chromatography (silica gel, 25% EtOAc/hexanes) to afford 5.18 g (63%) of the product as a dark red powder.

¹H NMR (400 MHz, DMSO-d₆) δ 11.72 (s, 1H), 11.34 (s, 1H), 7.43-7.31 (m, 2H), 7.10 (ddd, J = 8.2, 7.1, 1.2 Hz, 1H), 7.03 (ddd, J = 8.2, 7.1, 1.0 Hz), 2.41 (s, 3H).

¹³C NMR (100 MHz, DMSO-d₆) δ 169.85, 167.16, 139.52, 138.22, 135.60, 126.18, 121.30, 120.54, 120.11, 119.61, 111.06, 100.76, 13.72.

2.10.5.35 Synthesis of 3-bromo-2-methylbenzo[b]thiophene.

A solution of 2-methylthiaphthene (4.65 g, 3.14 × 10⁻² mol) in CHCl₃ (100 mL) at 0°C was treated drop wise over 30 min with a solution of bromine (1.78 mL, 3.14 × 10⁻² mol) in CHCl₃ (25 mL) using a dropping funnel. The cooling bath was removed and the reaction mixture was stirred at room temperature for 14 h. The reaction was washed with sodium thiosulfate (10% solution in water) and saturated sodium bicarbonate. The
aqueous layer was extracted with CHCl₃ (100 mL). The CHCl₃ layers were combined and washed with saturated sodium chloride (100 mL), dried over MgSO₄, filtered and concentrated under reduced pressure to afford a yellow oil. Purification by column chromatography (silica, 100% hexanes) afforded 6.1 g (86%) of the product as a clear, colorless oil, which solidified into colourless needles upon storing in a freezer.

¹H NMR (400 MHz, CDCl₃) δ 7.77-7.69 (m, 2H), 7.42 (ddd, J = 8.2, 7.2, 1.1 Hz, 1H), 7.34 (ddd, J = 8.1, 7.3, 1.2 Hz, 1H), 2.57 (s, 3H).

2.10.5.36 Synthesis of 3-(2-methyl-1H-indol-3-yl)-4-(2-methylbenzo[b]thiophen-3-yl)-1H-pyrrole-2,5-dione.

A solution of 3-bromo-2-methylbenzo[b]thiophene (1.07 g, 4.69 × 10⁻³ mol) in anhydrous THF (25 mL) was cooled to −60°C in a dry ice/acetone bath and then treated drop wise with n-BuLi (2.5 M solution in hexanes, 2.1 mL, 5.16 × 10⁻³ mol). The resulting solution was stirred at this temperature for an additional 20 min and then was treated with tributylborate (1.4 mL, 5.16 × 10⁻³ mol). After stirring at this temperature for 30 min, the reaction was allowed to warm to room temperature by removing the cooling bath. The resulting solution was added to a mixture of 3-bromo-4-(2-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (1.22 g, 3.99 × 10⁻³ mol), potassium carbonate (3.24 g, 2.35 × 10⁻² moles) and DME/H₂O (1.5:1, 100 mL) under nitrogen. The mixture was heated at reflux for 20 h, allowed to cool to room temperature, poured into water and extracted with EtOAc (2 × 100 mL). The organic layers were combined, washed with brine then with water, dried over MgSO₄, filtered and concentrated under reduced pressure to afford a dark red/orange solid. Purification by column chromatography (silica gel, 1–5% MeOH in CH₂Cl₂) afforded 0.61 g (41%) of the product as an orange powder.
\[ \text{Mp} = 205\text{-}210^\circ \text{C}. \]

\(^1\text{H} \text{ NMR} (400 \text{ MHz, CDCl}_3) \delta 11.44 \ (s, 1\text{H}), 11.11 \ (s, 1\text{H}), 7.83 \ (d, J = 7.9 \text{ Hz, 1H}), 7.38 \ (d, J = 7.8 \text{ Hz, 1H}), 7.25\text{-}7.14 \ (m, 3\text{H}), 7.05 \ (d, J = 7.9 \text{ Hz, 1H}), 6.99\text{-}6.94 \ (m, 1\text{H}), 6.78\text{-}6.74 \ (m, 1\text{H}), 2.14 \ (s, 3\text{H}), 2.00 \ (s, 3\text{H}). \]

\(^{13}\text{C} \text{ NMR} (100 \text{ MHz, CDCl}_3) \delta 172.13, 171.98, 141.17, 138.70, 138.68, 138.33, 137.82, 135.94, 130.01, 126.94, 124.61, 124.39, 123.95, 123.06, 122.43, 121.55, 119.98, 119.63, 111.22, 103.04, 15.40, 13.37.

HRMS (ESI): calculated for C\(_{22}\)H\(_{16}\)N\(_2\)O\(_2\)S (M\(+\)H): 373.0932, found: 373.0991.

UV-vis (EtOAc, 50 \(\mu\text{M})\): \(\lambda_{\text{max}} = 438 \text{ nm}, \varepsilon = 4800 \text{ M}^{-1}\text{cm}^{-1}.\)

\textbf{2.10.5.37 Synthesis of 1-\((3\text{-}(\text{dimethylamino)propyl})\)-3-(2-methyl-1H-indol-3-yl)-4-(2-methylbenzo[b]thiophen-3-yl)-1H-pyrrole-2,5-dione.}

Prepared based on literature procedure.\(^{116}\) A solution of 3-(2-methyl-1H-indol-3-yl)-4-(2-methylbenzo[b]thiophen-3-yl)-1H-pyrrole-2,5-dione (0.15 g, \(4.16 \times 10^{-4} \text{ mol}\)) in CH\(_3\)CN (10 mL) and THF (2.5 mL) was treated with Cs\(_2\)CO\(_3\) (0.67 g, \(2.10 \times 10^{-3} \text{ mol}\)) and KI (0.15 g, \(8.80 \times 10^{-4} \text{ mol}\)). The resulting suspension was stirred at room temperature for 30 min at which time it was treated with 3-dimethylamino-1-propyl chloride hydrochloride (0.16 g, \(1.02 \times 10^{-3} \text{ mol}\)). The reaction was heated at reflux for 3 h (the progress was monitored by TLC), allowed to cool to room temperature and poured into a separatory funnel, followed by the addition of EtOAc (50 mL) and H\(_2\)O (50 mL). The layers were separated and the water layer extracted with EtOAc (2 \times 50 \text{ mL}). The combined organic layers were washed with brine (2 \times 50 \text{ mL}), dried over MgSO\(_4\), filtered
and concentrated under reduced pressure to afford an orange oil. Purification by column chromatography (silica, 5% MeOH in CH₂Cl₂) afforded 0.16 g (89%) of the product as a bright orange/red powder.

Mp = 126-128°C.

¹H NMR (400 MHz, DMSO-d₆) δ 11.51 (s, 1H), 7.85 (d, J = 7.9 Hz, 1H), 7.40 (d, J = 7.9 Hz, 1H), 7.28-7.21 (m, 2H), 7.21-7.15 (m, 1H), 7.06 (d, J = 7.9 Hz, 1H), 7.01-6.95 (m, 1H), 6.78 (m, 1H), 3.64 (t, J = 7.0 Hz, 2H), 2.48-2.43 (m, 2H), 2.27 (s, 6H), 2.15 (s, 3H), 2.02 (s, 3H), 1.88-1.78 (m, 2H).

¹³C NMR (101 MHz, DMSO-d₆) δ 170.85, 170.74, 141.36, 138.91, 138.64, 137.85, 137.59, 135.99, 129.01, 126.88, 124.60, 124.46, 123.88, 123.10, 122.48, 121.65, 120.02, 119.66, 111.29, 103.09, 56.84, 45.15, 36.68, 25.92, 15.44, 13.41.


UV-vis (EtOAc, 50 µM): λₘₐₓ = 445 nm, ε = 6200 M⁻¹cm⁻¹.

2.10.5.38 Synthesis of the ring-closed isomer of 1-(3-(dimethylamino)propyl)-3-(2-methyl-1H-indol-3-yl)-4-(2-methylbenzo[b]thiophen-3-yl)-1H-pyrrole-2,5-dione

A 3 mL aliquot of a solution of 1-(3-(dimethylamino)propyl)-3-(2-methyl-1H-indol-3-yl)-4-(2-methylbenzo[b]thiophen-3-yl)-1H-pyrrole-2,5-dione (25 mg, 5.46 × 10⁻⁶ mol) in THF (25 mL) in a cuvette was irradiated with 450 nm light (slit width 10 nm) while stirring with a 1.5 mm x 8 mm stir-bar (~70 minutes). The THF was removed under reduced pressure (all manipulations were done in the dark), the dark red/purple residue was
dissolved in 2.5% MeOH in DCM and purified using column chromatography (silica, 2.5% MeOH in DCM), affording 37% of the ring-closed isomer.

\[^1\text{H} \text{NMR (400 MHz, DMSO-}d_6\text{)} \delta\ 9.22\ (\text{dd, } J = 8.1, 0.6 \text{ Hz, 1H}), 8.59-8.50\ (\text{m, 1H}), 7.48\ (\text{dd, } J = 7.9, 0.6 \text{ Hz, 1H}), 7.44-7.37\ (\text{m, 1H}), 7.32\ (\text{ddd, } J = 8.4, 7.2, 1.4 \text{ Hz, 1H}), 7.24\ (\text{ddd, } J = 8.3, 7.2, 1.2 \text{ Hz, 1H}), 6.93\ (\text{s, 1H}), 6.86-6.74\ (\text{m, 2H}), 3.62\ (\text{t, } J = 6.7 \text{ Hz, 2H}), 2.39-2.20\ (\text{s, 6H}), 1.79\ (\text{s, 2H}), 1.67\ (\text{s, 3H}), 1.62\ (\text{s, 3H}).\]

UV-vis (MOPS buffer, DMSO 1%, 50 µM): \(\lambda_{\text{max}} = 530 \text{ nm (}\varepsilon = 7200 \text{ cm}^{-1}\text{M}^{-1})\), 415 nm (\(\varepsilon = 9800 \text{ cm}^{-1}\text{M}^{-1}\)), 355 nm (\(\varepsilon = 7200 \text{ cm}^{-1}\text{M}^{-1}\)).

2.10.5.39 \textit{Synthesis of 3-(2-methyl-1H-indol-3-yl)-4-(2-methylbenzo[b]thiophen-3-yl)-1H-pyrrole-2,5-dione.}

Prepared based on literature procedure.\textsuperscript{115} Added NaH (0.13 g, 5.47 x 10\(^{-3}\) mol) to a Schlenk tube followed by THF (10 mL) added 3-(2-methyl-1H-indol-3-yl)-4-(2-methylbenzo[b]thiophen-3-yl)-1H-pyrrole-2,5-dione (0.20 g, 5.38 x10\(^{-4}\) mol) slowly over ~15 min (in an ice/water bath). Solution bubbled vigorously and changed from a yellow/orange suspension to a deep red/brown suspension. Let stir under N\(_2\) (0°C) for 30 min then added N,N'-dimethylamino propyl chloride (0.140 g, 8.86 x 10\(^{-4}\) mol). Heated to 70°C for ~24 hours, cooled to RT, quenched mixture with NH\(_4\)Cl (sat’d) and extracted with EtOAc. Combined the organic layers, dried over MgSO\(_4\), filtered and concentrated under reduced pressure, deep red oil. Purified crude material using column chromatography silica gel (Acetone with a few drops of TEA).
\(^1\)H NMR (400 MHz, DMSO) \(\delta\) 11.14 (s, 1H), 7.82 (d, \(J = 8.1\) Hz, 1H), 7.44 – 6.96 (m, 6H), 6.85 (s, 1H), 4.09 (t, \(J = 6.5\) Hz, 2H), 2.16 (s, 3H), 2.07 – 1.81 (m, 11H), 1.62 (s, 2H).

\(^{13}\)C NMR (101 MHz, DMSO-d\(_6\)) \(\delta\) 171.65, 171.42, 152.49, 137.35, 136.03, 130.36, 130.29, 125.66, 124.03, 123.91, 123.37, 122.56, 121.94, 121.19, 119.85, 119.35, 109.71, 102.83, 55.82, 55.35, 44.94, 30.69, 29.59, 26.96, 14.99, 11.44.

HRMS (ESI): calculated for \(\text{C}_{27}\text{H}_{28}\text{N}_3\text{O}_2\text{S}\) (M+1) 458.1902, found: 458.1907.

UV-vis (THF, 50 \(\mu\)M): \(\lambda_{\text{max}} = 445\) nm, \(\varepsilon = 6200\) M\(^{-1}\)cm\(^{-1}\).

2.10.5.40 Synthesis of the ring-closed isomer of 3-(2-methyl-1H-indol-3-yl)-4-(2-methylbenzo[b]thiophen-3-yl)-1H-pyrrole-2,5-dione.

![Chemical structure](image)

A solution of 3-(2-methyl-1H-indol-3-yl)-4-(2-methylbenzo[b]thiophen-3-yl)-1H-pyrrole-2,5-dione (10.0 mg, 2.2 \(\times\) 10\(^{-3}\) moles/L) in a mixture of 1:1 THF/hexanes (10 mL) was prepared and transferred to a scintillation vial containing a stir bar. The vial was irradiated with 450 nm light (fluorimeter source, slit width 10 nm) while stirring for a total of 70 min. The solvent was removed under reduced pressure and then re-dissolved in DCM / 2.5% MeOH and purified using column chromatography (silica gel, DCM / 2.5% MeOH), affording 2.1 mg of the ring-closed isomer (21% yield).

\(^1\)H NMR (400 MHz, DMSO) \(\delta\) 11.49 (s, 1H), 9.25 (d, \(J = 8.0\) Hz, 1H), 8.63 (d, \(J = 7.3\) Hz, 1H), 7.52 (d, \(J = 7.6\) Hz, 1H), 7.48 – 7.35 (m, 2H), 7.32 – 7.21 (m, 1H), 6.90 – 6.75 (m, 2H), 3.22 – 3.09 (m, 1H), 2.44 – 2.30 (m, 2H), 2.22 (s, 6H), 1.89 – 1.74 (m, 2H), 1.58 (s, 3H), 1.51 (s, 3H).
UV-vis (THF, 25 µM): λ = 359 nm (ε = 10478 cm⁻¹M⁻¹), 409 nm (ε = 9843 cm⁻¹M⁻¹), 556 nm (ε = 8931 cm⁻¹M⁻¹).
3 Design, Synthesis and Light Controlled Reactivity using a Pyridoxal 5’-Phosphate Mimic

Some of the work presented in this chapter is published in the following: Danielle Wilson and Neil R. Branda. Turning “On” and “Off” a Pyridoxal 5’-Phosphate Mimic Using Light. Angewandte Chemie International Edition 2012, 51, 5431-5434. The synthesis, physical characterization, photochemical characterization, NMR activity studies were designed and conducted by Danielle Wilson. The manuscript was co-written by Danielle Wilson and Neil R. Branda. Neil R. Branda designed the structure and project concept.41

3.1 Chapter Goals

The goals of this chapter are to use the light induced changes in the electronic properties of a diarylethene photoswitch to influence its reactivity towards substrate. The photoswitch design was inspired by the enzyme cofactor, Pyridoxal 5’-phosphate (PLP), which relies on electronic changes in its core structure and surrounding protein environment to achieve various different chemical transformations with a range of amino acid substrates. In particular, two of the critical functional groups on PLP that are believed to be important for catalysis, are an aldehyde reactive site and an electron withdrawing pyridinium that stabilizes negative charge generated during substrate transformations. In our hybrid photoresponsive PLP system, the two important functional groups are appended to the external 5,5’-positions of a diarylethene photoswitch, so that the degree of electronic communication and reactivity towards substrate can be reversibly modulated with light.

This chapter first includes a brief introduction to Pyridoxal 5’-phosphate, how it functions as a unique cofactor and the different transformations it is involved in. Next, the photoresponsive PLP mimic design is presented and how the light induced changes in the diarylethene family of photoswitches can be used to modify the electronic
connection between two appended groups. The results section details the synthesis, characterization, photochemical performance, and then lastly a demonstration of how the reactivity our of analogue can be tuned in real-time with light to effect the outcome of a PLP-type reaction.

Chapter 1 provided an overview of the different methods for regulation of biological activity using light controlled small molecules. The majority of examples described to date take advantage of the differences in the size, shape, or spatial positioning that occur in the structure of a photosensitive molecule with light irradiation. However, a technique that is less studied is the manipulation of the electronic differences in the photoswitch core to influence a biologically relevant transformation.

3.2 Introduction to Pyridoxal 5’-phosphate function and reactivity

3.2.1 Cofactor structure and function

Pyridoxal 5’-Phosphate (PLP), also known as the biologically active form of vitamin B₆ is a versatile enzyme cofactor responsible for basic metabolism in all organisms from bacteria to humans. The cofactor is relatively small in size, and together with the appropriate enzyme, functions to catalyze a diverse range of enzymatic reactions including transamination, racemization, decarboxylation, as well as numerous elimination and replacement type processes. The structure of PLP is illustrated in the figure below. In addition to functioning as a cofactor in conjugation with enzymes, PLP can also perform substrate conversion in absence of an enzyme. Although the non-enzymatically catalyzed reactions are not as efficient (they may require heat, acid/base etc) and often, are not selective for one process over another.
The two minimum essential functional groups that are important for PLP catalysis in non-enzymatically assisted transformations, are namely the aldehyde, where amino acid substrates react to undergo conversion and the pyridinium group, which is believed to play two crucial roles, stabilization of the negative charge generated after deprotonation of the $\alpha$-hydrogen of the amino acid through the quinoidal intermediate and also to decrease acidity of the $\alpha$-hydrogen making deprotonation favourable. The phenolic group helps to stabilize and orient the adjacent aldehyde group (or intermediate species generated at the 4-position on the ring) in an arrangement that lowers transition state energy for efficient deprotonation/protonation sequences or reaction with nucleophiles. The anionic phosphate group is important for anchoring the cofactor in the active site maintaining an optimal binding orientation in the enzyme for catalysis.

Despite the range of possible reaction pathways, all PLP catalyzed reaction proceed through a common Schiff base intermediate. An example of two different PLP mediated processes that diversify from the quinonoid species, transamination and racemization is given in Figure 3.2. In the first step, an amino acid reacts with the aldehyde site, to give the imine or Schiff base adduct as well as water. Due to the strong conjugation between the Schiff base with the electron withdrawing pyridinium group, the acidity of the $\alpha$-hydrogen of the amino acid is increased so that deprotonation can now occur more easily. The resulting carbanion, generated as a result of deprotonation, can be stabilized through resonance as the neutral quinoidal species, or quinonoid intermediate. Depending on the reaction conditions, the fate of the quinonoid differs. The figure below illustrates two possible reaction outcomes which vary in the position of the carbanion.
that re-protonation occurs at. In the racemization example, (pathway denoted by the pink arrows), the sp² hybridized C=N double bond of the amino acid is protonated. Depending on which face of the double bond protonation occurs at, either the D or L amino acid can be produced. In the transamination example, (pathway given by the orange arrows), the C=C double bond at the 4 position on the ring, is protonated. After hydrolysis, the α-keto acid is generated, along with pyridoximine 5'-phosphate.

Figure 3.2 – Proposed mechanism for PLP catalyzed transamination (orange coloured arrow pathway) and racemization (pink coloured arrow pathway) both proceeding through the resonance stabilized quinoidal intermediate.

In enzyme assisted PLP catalysis, the cofactor is covalently bound to the enzyme through an imine bond formed with a lysine residue in the active site, also referred to as the internal aldime, see Figure 3.3 for the structure of the active site bound PLP. The imine bond between the lysine can be displaced by substrate during catalysis.
Figure 3.3 - PLP bound in the active site of the enzyme, Alanine Racemase (PDB ID 1XFC).\textsuperscript{129} The cofactor is held in place in the active site by a combination of electrostatic and hydrogen bonds formed between the phosphate group, phenol and pyridinium. A covalent bond is formed with an active site lysine residue giving the internal aldimine.

The phosphate groups make a number of interactions with the amino acid side chains present in the active site, effectively orienting the cofactor in an ideal conformation for catalysis. The unique amino acid sequence in the binding site of PLP is what allows for high selectivity and stereospecificity of products. For example the Alanine Racemase enzyme shown in Figure 3.3 will have a different active site sequence than an alanine transaminase.

3.3 Photoresponsive PLP mimic design

Here we present a new PLP cofactor mimic whose substrate catalysis rates can be reversibly varied, when desired, by irradiation with light. By using the diarylethene photoswitch to separate the aldehyde and pyridinium, this creates a new way to tune the degree of communication between these essential groups, and translates into differences in reactivity. For synthetic ease, the phosphate, methyl, and phenolic groups were not included in the structure.
Catalysis should be possible without including these groups in our photochromic PLP design. Although, it should be noted that we expect a decrease in catalysis rates in the absence of the phenolic group. As mentioned earlier in the chapter, the phenolic group aligns the aldehyde group in the correct orientation for efficient deprotonation/protonation sequences or reaction with nucleophiles. Whereas we expect that the absence of the phosphate group should not have a substantial influence catalysis rate with our PLP mimic in our enzyme free studies. The phosphate groups are important for recognition and binding in the active site of the enzyme. Therefore, any experiments where enzyme binding is required, the phosphate group (or other related group) should be included.

3.3.1 Structure and predicted light induced electronic changes

The structural design and light induced photoisomerization of the proposed PLP cofactor mimic is shown in Scheme 3.1. As outlined in the previous section, the minimum requirement for substrate conversion in PLP is the presence of an aldehyde conjugated to a pyridinium. In PLP, the aldehyde is substituted directly on the pyridine ring, this ensures the two groups are in conjugation with one another. The incorporation of a DTE between these two essential groups, allows for modification of this conjugation. In the flexible ring open form, 3.1-o, the thienyl groups are both free to rotate around the central cyclopentadiene double bond, and therefore the communication between the aldehyde and pyridinium is weak as the two groups are electronically isolated from each other. Whereas switching the system to the conformationally restricted ring-closed form, 3.1-c, with UV light, the conjugation remains in tact, as there is extended electronic communication along the DTE backbone, highlighted in bold.
Scheme 3.1 - In Pyridoxal phosphate, the two essential groups for catalysis, highlighted in the orange boxes are electronically connected. In the photoresponsive PLP design (compound 3.1), a diarylethene is incorporated between the aldehyde and pyridinium, allowing for light induced changes in the electronic communication between them.

The extended communication achieved with the ring-closed isomer 3.1-c, can be expected to influence the catalysis ability of the cofactor mimic in several stages of the reaction. In the first step, the increased electrophilic character of the aldehyde site, should favour the formation of the imine condensation product over the ring-open isomer, which is not conjugated to the pyridinium. In the second stage of the reaction, the additional conjugation will help to increase the acidity of the amino acid α-hydrogen atom, and the carbanionic intermediate formed can be resonance stabilized through a quinonoid type intermediate, similar to PLP. Whereas the ring-open isomer shares similar characteristics as an aryl aldehyde, and is presumably comparatively less reactive towards amino acid substrate. Catalytic activity can therefore be controlled externally and reversibly using light.

![Scheme 3.1 Diagram](image-url)
Incorporating the DTE photoswitch into the structure of Pyridoxal phosphate, as we have proposed for compound 3.1, will undoubtedly reduce the reactivity of the cofactor in comparison to the natural cofactor. The reactive aldehyde site and the pyridinium are considerably further apart in 3.1-c with the introduction of the photoswitch, versus in PLP where the aldehyde is directly substituted on the pyridinium ring. Therefore the catalysis rates of 3.1-c are not expected to be comparable to PLP. The goal of this project is to demonstrate the ability to use light energy to reversibly influence the catalysis rates of a PLP type reaction through variation in the degree of communication between two groups required for catalysis. Compound 3.1 is also not designed to bind in the active site of PLP dependent enzymes and serve as a replacement cofactor for PLP in enzymatically assisted reactions.

3.4 Results and discussion

3.4.1 Synthesis

The synthesis of the target PLP mimic 3.1-o was performed using the steps listed in Scheme 3.2 and Scheme 3.3. The precursor diarylethene, 3.5, was prepared in multigram quantities in three steps according to a modified procedure described by Feringa et al.130 Starting with commercially available 2-methylthiophene, the 5-position on the thiophene ring was chlorinated with NCS in benzene and acetic acid to give 3.3, excellent yield. A Friedel-Crafts acylation was used to construct diketone 3.4, by reaction of glutaryl dichloride with two equivalents of 3.3 and AlCl₃. The crude product isolated from the reaction mixture was used directly in the next step without purification. The cyclopentadiene bridge was prepared using an intramolecular McMurry reaction, involving refluxing the diketone 3.5, in a THF suspension of zinc dust and TiCl₄.
Scheme 3.2 - Preparation of the diarylethene intermediate 3.5 starting from commercially available 2-methylthiophene.\textsuperscript{130}

Several routes were considered for the construction of the desired target 3.1. Initially we envisioned preparing the aldehyde derivative 3.6 and then using a Pd(0) catalyzed cross-coupling reaction directly from the thienyl chloride to yield 3.7, see Scheme 3.3.\textsuperscript{131} The installation of the aldehyde proceeded well, and was achieved through treatment of 3.5 with t-BuLi, followed by quenching with DMF and an acidic workup to yield 3.6. Although the cross-coupling reaction outlined in the last step of Scheme 3.3, between the thienyl chlorine of 3.6 and pyridinyl boronic acid, in the presence of an activating ancillary ligand typically yield insufficient amounts of product (<5%) for practical use later on.

Scheme 3.3 – Proposed route for preparation of asymmetric pyridine aldehyde 3.7 from the dichloro switch precursor 3.5.

Instead we examined an alternate route, involving replacement of the chlorine atoms of 3.5 with bromine atoms. This was achieved by treating 3.5 with t-BuLi in diethyl ether and subsequently quenching the lithiated intermediate with bromine drop wise, to
give the product 3.8 in 56% yield. The aldehyde derivative was prepared using the same conditions outlined for preparation of aldehyde 3.6, again the aryl halide 3.8 was treated with 1 equiv of t-BuLi in a lithium halogen exchange, and the resulting species quenched with DMF and hydrolyzed with dilute acid during the workup to yield 3.9.

Scheme 3.4 – Preparation of the target PLP mimic 3.1

A Suzuki coupling was used to attach the pyridine group and yield 3.7. In the final step the pyridinium salt was prepared by heating 3.7 with excess methyl p-toluenesulfonate in benzene for 2 days. The reaction gave the corresponding salt, 3.1-o in 87% yield, as vibrant yellow crystals (which quickly darkened to deep green crystals unless kept in the dark).

3.4.2 Photoswitchable Behaviour of 3.1

The photochemical properties of the target compound 3.1-o, were investigated by UV-visible absorption spectroscopy. When a solution of 3.1-o in THF, is irradiated with 365 nm light, two new bands appear in the absorption spectrum over time, an intense and relatively broad band appeared in the visible region of the spectrum, centered at ~640 nm and a second smaller band at 415 nm indicating conversion to the ring-closed isomer, 3.1-c, see Figure 3.5. A decrease in the intensity of the bands in the UV region at 375 nm and 324 nm from 3.1-o are also visible. The data is consistent with the
expected changes that occur with conversion to the ring-closed isomer \textbf{3.1-c}, which has an extended \(\pi\)-conjugated backbone and shifts the absorption to the visible region. Two isosbestic points are observed at 401 nm and 310 nm, suggesting that ring-closure occurs without appreciable degradation or side reactions. The solution changed from pale yellow / clear to blue with ring-closure.

![Chemical Structures](image)

\textbf{Figure 3.5} – (a) UV-visible absorption spectrum of \textbf{3.1-o} in acetonitrile (50 \(\mu\)M) after exposure to 365 nm light at sequential 2 second intervals for a total of 68 seconds. (b) Kinetic plot summarizing the changes in the absorbance value at \(\lambda_{\text{max}} = 630\) nm as the concentration of \textbf{3.1-c} increases over time.

In preparation for the light controlled PLP mimic activity studies (featured in the next section), the photochemical cycling behaviour of \textbf{3.1-o} was also evaluated in an acetic acid and water mixture (the same solvent conditions used for light controlled catalysis measurements). Fortunately, and unlike the indolylmaleimides featured in Chapter 2, the photoconversion yields of \textbf{3.1-o} is not strongly dependent on the solvent polarity. Irradiation of compound \textbf{3.1-o} with 365 nm triggered a dramatic change in the
colour solution from pale yellow (almost clear) to a vibrant blue colour (Figure 3.6 (a)). Changes in the UV-visible absorption spectrum included appearance of an intense band at ~640 nm and a smaller band at 415 nm, whereas the in the UV region, while the spectral features at 274 nm, 325 nm, and 373 nm decrease in intensity. The photostationary state is reached after ~105 seconds under these conditions, (50 µM, 365 nm light source from a handheld UV lamp).

![Figure 3.6](image)

**Figure 3.6** – (a) Irradiation of a solution of 3.1-o in CH₃CO₂H/H₂O (9:1) with 365 nm light produces the ring-closed isomer 3.1-c, this is accompanied by a change in the solution colour from pale yellow to blue (b) Changes in the UV-visible absorption spectrum of 3.1-o in CH₃CO₂H/H₂O (9:1) (50 µM) after exposure to 365 nm light at sequential 5 second intervals for a total of 68 seconds to give 3.1-c. (c) Kinetic plot summarizing the changes to the absorbance at λ_{max} = 640 nm, as the concentration of 3.1-c increases over time with 365 nm irradiation. (d) Summary of the changes that occur in the absorbance at 375 nm (from 3.1-o) and 640 nm (from 3.1-c) during alternate irradiation with 365 nm light for 105 seconds to induce ring-closure, followed by light > 490 nm for 60 seconds to induce cycloreversion, for a total of 10 cycles.

The ring-closed isomer 3.1-c is stable at room temperature in the dark and can be converted back to the ring-open isomer with exposure to light >490 nm. The reversibility of the photoswitching process was also investigated by alternating exposure of the sample solution to UV light (365 nm for 105 seconds) and visible light (>490 nm
for 60 seconds) for a total of 10 cycles. There does appear to be some photochemical degradation, based on the decreasing intensity of the absorbance at 375 nm and 540 nm with successive cycling. However, for the purposes of our light controlled catalyst, extensive cycling from 3.1-o to 3.1-c is not anticipated to be necessary.

3.4.3 NMR spectroscopy

The photocyclization process of 3.1-o was evaluated using NMR spectroscopy, to confirm the structure of 3.1-c and determine the approximate yield of the reaction. A solution of the ring-open isomer 3.1-o (5000 µM in CD$_3$CO$_2$D/D$_2$O) in a glass NMR tube was irradiated with 365 nm light for 30 minutes at which point the photostationary state was reached. Irradiation time beyond 30 minutes did not yield significant changes to the spectrum. The $^1$H NMR spectrum recorded before and after light irradiation, and the changes in the region 12.0-6.5 ppm are provided in Figure 3.7.
The thienyl protons, labelled 2 and 3 in 3.1-o, underwent the largest change in chemical shift after cyclization. Initially, these appear at 7.83 ppm and 7.66 ppm in the ring-open isomer, 3.1-o. Conversion to the ring-closed isomer 3.1-c, disrupts the aromaticity of the thiophene π-system, which causes a measurable upfield shift the protons labelled 2’ and 3’ in the figure, to 7.32 ppm and 6.98 ppm, respectively. The remaining protons in the aromatic region change to a smaller extent with ring-closure, <0.1 ppm. Interestingly, the aldehyde peak, labelled position 1 and 1’ in Figure 3.7, shows a difference of ~0.07 ppm between the ring-open and ring-closed isomer, with the 3.1-c slightly more deshielded than 3.1-o. This observation was somewhat surprising considering that the aldehyde peak in the ring-closed isomer is expected to exhibit a

Figure 3.7 – Overlap of the $^1$H NMR spectrum from 12.0-6.5 ppm of the ring-open isomer 3.1-o (bottom) and the photostationary state mixture (top) containing 97% of 3.1-c (and the remaining 3% assigned to 3.1-o).
more prominent change in electron density with conjugation to the electron withdrawing pyridinium.

The photoconversion yield can be estimated from the relative integration of the peaks assigned to the ring-open and ring-closed isomers in the $^1$H NMR spectrum of the photostationary state mixture. Using Equation 1.1 and the integration of the aldehyde peak for the ring-closed and ring-open isomers, the photostationary state was estimated to be $\sim 97\%$ (see Figure 3.8).

![NMR Spectra](image)

**Figure 3.8** – Selected region of the $^1$H NMR spectrum (CD$_3$CO$_2$D/D$_2$O (9:1), 400 MHz) of the photostationary state mixture obtained from irradiation of 3.1-o with (365 nm light for 30 minutes). Using the ratio of the integrals from the aldehyde peak of 3.1-c and 3.1-o the photostationary state is estimated to be 97%.

### 3.4.4 Light Controlled Reactivity

The light controlled activity of 3.1 was studied using by $^1$H NMR spectroscopy. A proton-deuterium exchange reaction was used to compare the difference in ability of 3.1-o and 3.1-c to perform catalytic racemization of an amino acid (see Scheme 3.5). In this experiment, optically pure L-alanine and photoswitchable catalyst, either 3.1-o or 3.1-c
were dissolved in a mixture of D$_2$O and acetic acid-d$_4$ (9:1). A catalyst loading of 0.2 equivalents of either 3.1-o or 3.1-c were used during the exchange studies. The rate of deuterium exchange was monitored \textit{in situ} through $^1$H NMR spectroscopy by recording the decrease in integration of the $\alpha$-hydrogen peak of L-alanine over time as it is converted to $\alpha$-deuterium.$^{132}$

![Scheme 3.5 - A solution of optically pure L-alanine and 0.2 equiv of catalyst, either 3.1-o or 3.1-c in CD$_3$CO$_2$D/D$_2$O (9:1) was kept at constant temperature of 40°C (± 2°C) in a sealed NMR tube. The deuteration/racemization progress was monitored by recording the decrease in integration of the $\alpha$-hydrogen as it is converted to deuterium.](image)

In a study done by Matsuo \textit{et al}$^{132}$ comparing rates of deuteration and racemization in a series of amino acid derivatives, they concluded that the rates of deuterium exchange are nearly equal to the rates of racemization provided the sample is relatively dilute. The equation used to calculate the relative percentage deuterium-hydrogen exchanged during the $^1$H NMR experiment.$^{132}$

\[ \alpha D' = \left(1 - \frac{\alpha H^t}{\alpha H^0} / \frac{\text{ref} H^t}{\text{ref} H^0}\right) \times 100\% \]

\textit{Equation 3.1}

Where $\alpha H^t$/$\text{ref} H^t$ is the ratio of integration under the peaks corresponding to the $\alpha$-hydrogen ($\alpha$H) versus a non-exchangeable reference proton (refH) at a given time, and $\alpha H^0$/$\text{ref} H^0$ is the ratio at the initial $\alpha$-hydrogen integration at $t = 0$ hours.
We can similarly predict that proton-deuterium exchange rates reflect rates of racemization. This is a reasonable postulation, considering that once deprotonation of the chiral aldimine occurs, the resultant sp² hybridized intermediate is achiral, and assuming a similar mechanism as PLP, we can suspect there is an equal chance of reprotonation on either face of the double bond therefore giving a racemic mixture of D and L alanine as the product. See Figure 3.9 for an illustration of the mechanism involved in the light activated catalytic cycle.

Figure 3.9 – Proposed mechanism for racemization and proton-deuterium exchange catalyzed by the active ring-open isomer 3.1-c. The amino group of alanine reacts with the aldehyde to generate the corresponding aldimine product. Deprotonation gives the quinoidal intermediate. Reprotonation of the C=N double bond regenerates the D/L aldimine, then hydrolysis in the presence of D₂O gives alanine as a racemic mixture.
A partial $^1$H NMR spectrum is illustrated in Figure 3.10 for 3.1-o (a) and 3.1-o(b) demonstrating the changes in the $\alpha$-hydrogen peak of L-alanine at three different time points (start of the reaction $t = 0$ h (top panel), $t = 140$ h (middle panel) and at $t = 20$ days (bottom panel) during the experiment. With 3.1-o as the catalyst, the intensity of the quartet remains unchanged throughout the duration of the experiment indicating that very little deuterium exchange has taken place. However with 3.1-c, $\alpha$-hydrogen peak of L-alanine decreases in intensity over time, as the proton-deuterium exchange reaction proceeds.
Figure 3.10 – Partial $^1$H NMR spectrum (CD$_3$CO$_2$D/D$_2$O 9:1) for 3.1-c (a) and 3.1-o (b) at $t = 0$, $t = 140$ h and $t = 20$ days. The purple dotted line is drawn to highlight the maximum intensity of the alanine quartet.

A plot of the percentage exchange versus time is constructed in Figure 3.11 (b) and confirms that there is little racemization of L-alanine by the inactive isomer 3.1-o over time, less than 3% (orange trace in Figure 3.11 (b)). The conversion rate of 3.1-o was similar to the control study, containing no catalyst at all (gray trace in Figure 3.11 (b)) which yielded essentially 0% exchange. Whereas upon activation, 3.1-c shows a dramatic increase in the rate of deuterium exchange, reaching nearly 30% conversion over the same time period. There is a clear difference in the intensity of the $\alpha$-hydrogen quartet over time as shown below in Figure 3.11 (b) (blue trace). The proton-deuterium exchange progress appears to increase linearly with time until after ~80 h of heating at 40 °C, where the rate of exchange begins to slow. This is likely from a build up of product, and not from the ring-opening of 3.1-c at 40 °C, which would give the inactive isomer, 3.1-o, and potentially contribute to a slowed reaction rate. Compound 3.1-c is stable under the reaction conditions, including heating at 40 °C. The methyl group signal, (not shown) of L-alanine is converted from a doublet to a singlet as deuterium exchange proceeds.
Figure 3.11 (a) Proton-deuterium exchange experiment using L-alanine in the presence of either the ring-open isomer 3.1-o or the ring-closed isomer 3.1-c. (b) Plot of the percentage of deuterium exchange versus time. (c) Changes in percentage deuterium exchange over time in response to alternating cycles of irradiation with UV and visible light.

We also looked at the ability to reversibly switch deuterium exchange ‘on’ and ‘off’ by alternating illumination with UV and visible light, displayed in Figure 3.11 (c). Starting with the ring-open isomer 3.1-o, there is little racemization activity over time. After exposure of the sample to UV light, generating 3.1-c isomer, the activity increased dramatically. Exposure of this same sample to wavelengths >450 nm recovers the ring open isomer, and as expected, racemization is slowed again. The catalyst activity does not display complete on/off switching as the ring open isomer 3.1-o does racemize a small percentage of L-alanine. Production of both L and D-alanine isomers were later confirmed. Addition of a chiral shift reagent readily produces two well resolved diastereomeric complexes with unique chemical shifts in $^1$H NMR spectroscopy, confirming that racemization of the L-alanine has taken place as predicted, Figure 3.12.
(a). Controls containing pure L-alanine or D-alanine in the presence of the chiral shift agent are shown in 3.12 (b) and (c) respectively.

Figure 3.12 - Partial $^1$H NMR spectra (400 MHz, D$_2$O) of (a) the alanine (5.7 × 10$^{-2}$ M) isolated after reaction with 3.1-c performed in CD$_3$CO$_2$D/D$_2$O (9:1 v/v) (bottom), and the addition of 0.1 molar equivalent of the sodium [[(R)-1,2-diaminopropane-$N,N,N',N'$-tetraacetato]samarate (III) (top). (b) optically pure L-alanine (7.5 × 10$^{-2}$ M) (bottom), the same solution with the addition of 0.1 molar equivalent of the sodium [[(R)-1,2-diaminopropane-$N,N,N',N'$-tetraacetato]samarate (III) (middle), and the addition of a drop of a similar D-alanine solution (7.8 × 10$^{-2}$ M) (bottom) and (c) optically pure D-alanine (7.8 × 10$^{-2}$ M) (bottom), the same solution with the addition of 0.1 molar equivalents of the sodium [(R)-1,2-diaminopropane-$N,N,N',N'$-tetraacetato]samarate (III) (middle), and the addition of a drop of a similar solution of L-alanine (7.5 × 10$^{-2}$ M) (bottom). All samples were basified to pH 10–11 with NaOD (40% w/w in D$_2$O) to ensure binding to the chiral shift reagent. The inconsistencies in the chemical shifts between samples is due to the high pH sensitivity of the coordination to the chiral shift reagent.

In summary, the relative rates of deuterium exchange reflect the differences in electronic properties of the ring-open and ring-closed forms. Exchange is faster in 3.1-c. This supports the concept that the pyridinium is able to communicate through the DTE backbone with the aldehyde. The faster rate of exchange could be due to 1) increased aldehyde electrophilicity or 2) increased α-hydrogen acidity in the aldimine adduct formed with L-alanine (the pyridinium acts as an electron sink to stabilize negative charge build-up). This provides evidence for a dramatic increase in activity when in the ring-closed form, and offers new insights into controlling enzymatic processes by
modifying appropriate cofactors with photoresponsive units. Although rates of racemization are much slower than those catalyzed by enzymes. Typical racemization rates for enzyme assisted PLP conversions are several orders of magnitude ($10^4$-$10^5$) higher than rates possible with our PLP mimic. The system demonstrated in this chapter is the first example of switchable PLP mimic that can catalyze racemization. The results provide evidence that there is a stronger degree of communication between the pyridinium and aldehyde in the ring-closed form 3.1-c, and that the activity of the cofactor mimic can be effectively switched on and off with >20 fold difference in reaction rates between the two systems.

### 3.5 Conclusions

The chapter described the rational design, synthesis and evaluation of light modulated catalytic ability of a photoresponsive diarylethene-PLP hybrid. The system was inspired by the abundant enzyme cofactor, Pyridoxal 5’-phosphate (or vitamin B₆). This highly versatile catalyst that is responsible for a wide range of different transformations during amino acid metabolism. Variations in the cofactor’s electronic properties are what allow it to perform such a large number of catalytic conversions with a high degree of specificity. The structure consists of an aldehyde group, where the substrate undergoes conversion, and a pyridine group. The pyridine can undergo a series of protonation and deprotonation sequences, and the protonation state is what is used to influence the electronic nature of the cofactor and the outcome of a reaction with a substrate.

By incorporating a light responsive diarylethene into a PLP mimic’s structure, the electronic nature of the DTE-cofactor hybrid was influenced using light. The chapter demonstrates how positioning of the aldehyde and pyridinium groups on opposite sides of the diarylethene core, can be used to control the electrophilic properties of the aldehyde group and its reactivity towards an amino acid substrate. One photoisomer, the ring-closed 3.1-c, possesses extended electronic communication between the reactive site, which enhanced the electrophilicity of the aldehyde and the rate of substrate conversion ~20-fold in comparison to the ring-open isomer 3.1-o upon reaction with alanine. The second photoisomer, the ring-open isomer, showed very little substrate
conversion under the same conditions. This was expected, considering there is limited conjugation with the electron withdrawing group. The light induced changes in the photoresponsive cofactor mimic can be conveniently and reversibly switched from the inactive state, 3.1-o to the active state 3.1-c in situ, by exposure to UV or visible light.

Unfortunately, the cofactor system designed in this chapter, catalyzes substrate conversion at a rate that is much slower than the native cofactor, PLP, and would not be a suitable replacement for PLP in an enzymatic and non-enzymatic conversions for a number of reasons. The addition of the photoresponsive group increases the distance between the pyridinium and aldehyde, and this results in a much smaller change in electrophilicity of the aldehyde. By comparison, in PLP, the aldehyde is appended directly onto the pyridinium ring. Our design also does not include hydroxyl or phosphate groups. The hydroxyl group, substituted ortho to the aldehyde group, is important for aligning the aldehyde in the correct orientation for deprotonation, as well as, for reaction with substrate or incoming nucleophiles. The addition of the DTE group alters the size and shape of the photoresponsive PLP mimic. More work is required to design a photoresponsive PLP mimic, that is compatible for use as a suitable cofactor replacement.

3.6 Experimental

3.6.1 General.

Solvents used for synthesis were dried and degassed by passing them through steel columns containing activated alumina under nitrogen using an MBraun solvent purification system with the exception of THF, which was freshly distilled from sodium benzophenone under nitrogen prior to use. Solvents for NMR analysis were purchased from Cambridge Isotope Laboratories and used as received. All reagents were purchased from Aldrich or TCI America and were used as received. 1,2-Bis(5-chloro-2-methylthiophen-3-yl)cyclopent-1-ene 3.5 was prepared according to literature procedures. Column chromatography was performed using silica gel 60 (230-400 mesh) purchased from Silicycle Inc.
3.6.2 Instrumentation.

$^1$H and $^{13}$C NMR characterizations were performed on a Bruker Avance-400 instrument operating at 400.13 MHz for $^1$H NMR and 100.61 MHz for $^{13}$C NMR. Chemical shifts (δ) are reported in parts per million relative to tetramethylsilane using the residual solvent peak as a reference standard. Coupling constants (J) are reported in Hertz. FT-IR measurements were performed using a Nicolet Nexus 670 instrument. UV-Vis absorption spectroscopy was performed using a Varian Cary 300 Bio spectrophotometer. Low-resolution mass spectrometry measurements were performed using a Varian 4000 GC/MS/MS with electron impact operating at 10 mA or chemical ionization as the ionization source. High resolution mass spectrometry (HRMS) measurements were performed using an Agilent 6210 TOF LC/MS.

3.6.3 Photoinduced ring-closing and ring-opening reactions.

The ring-closing reactions of all compounds were carried out using the light source from a lamp used for visualizing thin-layer chromatography (TLC) plates at 365 nm (Spectroline E-series, 470 mW cm$^{-2}$). The ring-opening reactions were conducted using a 150 W tungsten source passed through a > 490 nm cut-off filter.

3.6.4 General procedure for hydrogen-deuterium exchange of L-alanine using 3.1-o or 3.1-c.

Compounds 3.1-o and 3.1-c were prepared for kinetic analysis from a stock solution of 3.1-o (18 mg, 0.03 mmol) dissolved in 1.62 mL of CD$_3$CO$_2$D and 0.18 mL of D$_2$O. Two 600 µL aliquots of this stock solution were measured and placed into separate glass vials. One of the solutions was irradiated with 365 nm light to convert 3.1-o to its ring-closed counterpart 3.1-c (photostationary state as confirmed by $^1$H NMR, 97% 3.1-c) which took approximately 120 min at this high concentration. The second solution was irradiated with visible light for approximately 2 min (150 W tungsten light passed through a 490 nm cut-off to filter higher energy light) to convert any traces of 3.1-c back to 3.1-o. Solid L-alanine (5 mg, 0.06 mmol) was added to each solution and mixed thoroughly to ensure complete dissolution. A 550 µL aliquot of each solution was transferred to two separate NMR tubes and sealed. The tubes were heated at 40°C using a temperature
controlled oil bath for the appropriate period of time and $^1$H NMR measurements were taken periodically. The tubes containing 3.1-c and 3.1-o were covered with aluminum foil to prevent isomerization by the lights in the lab. Control experiments were prepared in a similar manner as described above. L-Alanine (11 mg, 0.12 mmol) was dissolved in 540 µL of CD$_3$CO$_2$D and 60 µL of D$_2$O and 550 µL of this solution was placed in an NMR tube and sealed. The NMR tube was heated at 40°C using a temperature controlled oil bath and $^1$H NMR measurements were taken periodically. The integrations under the peaks for the chemical shifts corresponding to the $\alpha$-hydrogen of L-alanine in all spectra were normalized to the integrations under the peaks corresponding to the –CH$_3$ hydrogens of the tosylate counter ions, which was used as the internal standard. The percentage of hydrogen-deuterium exchange was calculated using the following equation:

$$\alpha D' = \left(1 - \frac{\alpha H'/\text{ref} H'}{\alpha H/\text{ref} H} \right) \times 100\%$$

Where $\alpha H'/\text{ref} H'$ is the ratio of integration under the peaks corresponding to the $\alpha$-hydrogen ($\alpha H$) versus a non-exchangeable reference proton (refH) at a given time, and $\alpha H/\text{ref} H$ is the ratio at the initial $\alpha$-hydrogen integration at t = 0 hours.

### 3.6.5 General procedure for analyzing samples of the amino acid with chiral shift NMR reagent.

L- and D-alanine were dissolved in D$_2$O in separate glass vials and basified to pH 10–11 with NaOD (40% w/w in D$_2$O). The chiral shift reagent, sodium [(R)-1,2-diaminopropane-$N,N,N',N'$-tetraacetato]samarate (III), (0.1 molar equivalent) was added to each solution, which was mixed thoroughly and placed into an NMR tube for analysis. Samples containing deuterium exchanged alanine ($\alpha$-deuterium) were prepared as described above by reaction in a mixture of CD$_3$CO$_2$D/D$_2$O, except were allowed to react for an additional 90 h at 55°C, with periodic exposure of NMR tube to 365 nm light to allow the racemization to proceed further before isolation.
3.6.6 Synthetic Procedures

3.6.6.1 Synthesis of 1,2-bis(5-bromo-2-methylthiophen-3-yl)cyclopent-1-ene

A solution of 1,2-bis(5-chloro-2-methylthiophen-3-yl)cyclopent-1-ene (2) (0.90 g, 2.74 mmol) in anhydrous diethyl ether (75 mL) in a flame dried three necked flask equipped with a mechanical stirrer was cooled to −60 °C (dry ice/acetone bath) and treated with t-butyllithium (1.7 M in pentane, 3.40 mL, 5.78 mmol) drop wise over a 15 min period under an atmosphere of nitrogen. After the addition, the mixture was stirred at −60 °C for an additional 10 min, at which time it was treated with a solution of bromine (0.31 mL, 6.05 mmol) in anhydrous Et₂O (10 mL) using a syringe. After stirring at −60 °C for 30 min, the cooling bath was removed and the reaction was allowed to slowly warm to room temperature. The reaction was poured into water, the layers were separated, and the organic layer washed with brine (2 × 50 mL). The organic layer was collected, dried over MgSO₄, filtered and the solvent removed under vacuum to yield a yellow oil. Purification using column chromatography (silica gel, hexanes) afforded 0.65 g (56%) of the product as a white crystalline solid.

M.p. = 85–90°C.

¹H NMR (400 MHz, CDCl₃) δ 6.71 (s, 2H), 2.72 (t, J = 8.0 Hz, 4H), 2.06–1.98 (q, J = 8.0 Hz, 2H), 1.88 (s, 6H).

¹³C NMR (100 MHz, CDCl₃) δ 136.2, 136.0, 134.3, 130.4, 107.4, 38.4, 22.8, 14.2 ppm.


IR (KBr pellet) = 2915, 1453, 990 cm⁻¹.
3.6.6.2 Synthesis of 4-(2-(5-bromo-2-methylthiophen-3-yl)cyclopent-1-en-1-yl)-5-methyl-thiophene-2-carbaldehyde

A solution of 1,2-bis(5-bromo-2-methylthiophen-3-yl)cyclopent-1-ene (3) (0.59 g, 1.41 mmol) in anhydrous THF (50 mL) in a flame dried three necked flask equipped with a mechanical stirrer was cooled to −60 °C (dry ice/acetone bath) and treated with t-butyllithium (1.7 M in pentane, 0.85 mL, 1.45 mmol) over a 15 min period. The reaction was allowed to stir for an additional 10 min at −60 °C and the metal-halogen exchange progress was monitored by TLC (1:1 hexanes/ethyl acetate). After the starting material was consumed, the reaction mixture was treated with DMF (0.22 mL, 2.85 mmol) and stirred at −60 °C for 1 h. The cooling bath was removed and the reaction was allowed to slowly warm to room temperature. The mixture was poured into 2M HCl (50 mL) and extracted with Et₂O. The Et₂O layer was washed with saturated aqueous NaHCO₃ (2 × 50 mL), followed by brine (2 × 50 mL). The ether layer was dried over MgSO₄, filtered and concentrated under vacuum to give a yellow oil. Purification by column chromatography (silica, 20:1 hexanes/EtOAc) afforded 0.36 g (69%) of the product as a pale yellow oil.

¹H NMR (400 MHz, CDCl₃) δ 9.74 (s, 1H), 7.43 (s, 1H), 6.71 (s, 1H), 2.81–2.74 (m, 4H), 2.08 (s, 3H), 2.11–2.03 (m, 2H), 1.85 (s, 3H).

¹³C NMR (CDCl₃ 100 MHz) δ 182.6, 146.6, 140.1, 137.9, 137.6, 135.8, 134.0, 133.9, 130.4, 126.6, 125.8, 38.5, 38.4, 23.0, 15.5, 14.2.


IR (NaCl, neat): 2956, 2842, 1666, 1451, 1143 cm⁻¹.
3.6.6.3 Synthesis of 5-methyl-4-(2-(2-methyl-5-(pyridin-4-yl)thiophen-3-yl)cyclopent-1-en-1-yl)thiophene-2-carbaldehyde.

A mixture of K$_2$CO$_3$ (0.53 g, 3.83 mmol), DME (30 mL) and water (20 mL) in a three necked flask was purged with N$_2$ for 0.5 h. The mixture was treated with 4-(2-(5-bromo-2-methylthiophen-3-yl)cyclopent-1-en-1-yl)-5-methyl-thiophene-2-carbaldehyde (4) (0.28 g, 0.75 mmol), pyridinyl boronic acid (0.14 g, 1.14 mmol) and tetrakis(triphenylphosphine)palladium(0) (0.08 g, 0.07 mmol). The reaction was heated at reflux under nitrogen for 18 h, at which time it was poured into water and extracted with CH$_2$Cl$_2$ (2 × 25 mL). The organic phases were combined, dried over MgSO$_4$, filtered and concentrated under vacuum to yield a brown oil. Purification by column chromatography (silica, 3:1 hexanes/EtOAc) afforded 0.21 g (74%) of the product as a white fluffy solid.

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 9.74 (s, 1H), 8.55 (d, $J$ = 5.1 Hz, 2H), 7.46 (s, 1H), 7.34 (d, $J$ = 5.1 Hz, 2H), 7.17 (2H, 1H), 2.87–2.82 (m, 4H), 2.16–2.10 (m, 2H), 2.09 (s, 3H), 1.98 (s, 3H).

$^{13}$C NMR (CDCl$_3$ 100 MHz) $\delta$ 182.5, 150.3, 146.5, 141.2, 140.0, 137.8, 137.5, 137.3, 137.0, 136.7, 135.9, 133.9, 126.0, 119.3, 38.5, 38.3, 22.9, 15.5, 14.6.


IR (CCl$_4$): 3041, 2964, 2850, 1642, 1549, 1452, 1246, 1049 cm$^{-1}$.
3.6.6.4 Synthesis of bis(4-(4-(2-(5-formyl-2-thiophen-3-yl)cyclopent-1-en-1-yl)-5-methylthiophen-2-yl)-1-methylpyridin-1-ium) mono(4-methylbenzenesulfonate).

A solution of methyl p-toluenesulfonate (0.15 g, 0.81 mmol) and 5-methyl-4-(2-(2-methyl-5-(pyridin-4-yl)thiophen-3-yl)cyclopent-1-en-1-yl)thiophene-2-carbaldehyde (5) (0.09 g, 0.25 mmol) in benzene (25 mL) was heated at reflux until consumption of the starting material was observed by TLC (2:1 hexanes/EtOAc), approximately 48h. The benzene was removed under reduced pressure to yield a dark green solid. Any excess methyl p-toluenesulfonate was removed by washing the resulting green solid several times with Et₂O, yielding 0.12 g (87%) of the product as a green microcrystalline powder.

M.p. = 104–108°C.

¹H NMR (400 MHz, CDCl₃) δ 9.75 (s, 1H), 8.92 (d, J = 6.4 Hz, 2H), 7.82 (d, J = 6.4 Hz, 2H), 7.78 (d, J = 7.8 Hz, 2H), 7.58 (s, 1H), 7.44 (s, 1H), 7.13 (d, J = 7.7 Hz, 2H), 4.43 (s, 3H), 2.85 (m, 4H), 2.32 (s, 3H), 2.18-2.12 (m, 2H), 2.11 (s, 3H), 2.02 (s, 3H).

¹³C NMR (CDCl₃ 100 MHz) δ 182.5, 148.2, 146.3, 145.5, 144.4, 143.5, 140.2, 139.4, 138.9, 137.6, 137.1, 135.5, 134.8, 133.0, 132.2, 128.7, 125.9, 121.7, 47.6, 38.5, 38.4, 22.9, 21.3, 15.5, 15.0.


IR (KBr pellet): 3052, 2952, 2843, 1662, 1639, 1553, 1455, 1192, 1122, 1033, 1010 cm⁻¹.
3.6.6.5 Synthesis of the ring closed isomer of bis(4-(4-(2-(5-formyl-2-methylthiophen-3-yl)cyclopent-1-en-1-yl)-5-methylthiophen-2-yl)-1-methylpyridin-1-ium) mono(4-methylbenzenesulfonate).

A solution of the ring-open isomer 3.1-o in CD$_3$CO$_2$D (5 × 10$^{-3}$ M) was used for the $^1$H NMR experiments to determine the content of each isomer at the photostationary state. The solution was added to a NMR tube and irradiated with 365 nm light until no further changes were observed in the NMR spectrum (~30 min total).

$^1$H NMR (400 MHz, CD$_3$CO$_2$D) δ  $^1$H NMR (400 MHz, acetic acid-d$_6$) δ 9.87 (s, 1H), 8.85 (d, $J$ = 6.9 Hz, 2H), 8.10 (d, $J$ = 6.9 Hz, 2H), 7.81 (d, $J$ = 8.2 Hz, 3H), 7.41 (s, 1H), 7.30 (d, $J$ = 8.1 Hz, 3H), 7.07 (s, 1H), 4.50 (s, 3H), 2.77 – 2.66 (m, 4H), 2.44 (s, 3H), 2.17 (s, 4H), 2.09 – 2.02 (m, 2H). *one of the methyl group (-CH$_3$) resonances overlaps with the solvent peak (acetic acid, at 2.14-2.12 ppm).
References


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Appendix A.

Chapter 2 – NMR Spectroscopy

\[ ^1H \text{NMR} \ (500 \text{ MHz, Acetone}) \delta \ 7.99 \ (d, J = 6.4, 2.8 \text{ Hz, } 1H), \ 7.50 – 7.40 \ (m, 1H), \ 7.28 – 7.18 \ (m, 2H), \ 3.98 \ (s, 3H), \ 2.68 \ (s, 3H). \]

Figure A.2.1 – \(^1\)H NMR of methyl 2-(2-methyl-1H-indol-3-yl)-2-oxoacetate (500 MHz, acetone-d\(_6\)).

\[ ^1H \text{NMR} \ (500 \text{ MHz, CDCl}_3) \delta \ 10.03 \ (s, 1H), \ 7.58 – 7.55 \ (m, 3H), \ 7.41 – 7.37 \ (m, 2H), \ 7.33 – 7.29 \ (m, 1H), \ 7.26 \ (s, 2H), \ 2.81 \ (s, 3H). \]

Figure A.2.2 – \(^1\)H NMR of 2.2-methyl-5-phenylthiophene-3-carbaldehyde (500 MHz, CDCl\(_3\)).
Figure A.2.3 – $^1$H NMR of (2-methyl-5-phenylthiophen-3-yl)methanol (500 MHz, CDCl$_3$).

$^1$H NMR (500 MHz, CDCl$_3$) δ 7.57 – 7.52 (m, 2H), 7.38 – 7.33 (m, 2H), 7.29 – 7.25 (m, 2H), 7.18 (s, 1H), 4.47 (s, 2H), 2.46 (s, 3H).

Figure A.2.4 – $^1$H NMR of 3-(bromomethyl)-2-methyl-5-phenylthiophene (500 MHz, CDCl$_3$).
$^1$H NMR (400 MHz, CDCl$_3$) δ 7.59 – 7.53 (m, 2H), 7.43 – 7.35 (m, 2H), 7.34 – 7.27 (m, 1H), 7.18 (s, 1H), 3.82 (s, 2H), 2.45 (s, 3H).

Figure A.2.5 – $^1$H NMR of 2-(2-methyl-5-phenylthiophen-3-yl)acetonitrile (400 MHz, CDCl$_3$).

$^{13}$C NMR (101 MHz, CDCl$_3$) δ 141.39, 135.54, 133.87, 129.05, 127.72, 125.87, 125.60, 123.84, 123.83, 117.55, 17.08, 13.26.

Figure A.2.5 – $^{13}$C NMR of 2-(2-methyl-5-phenylthiophen-3-yl)acetonitrile (101 MHz, CDCl$_3$).
$^1$H NMR (500 MHz, CDCl$_3$): 5.75 – 7.51 (m, 2H), 7.36 – 7.32 (m, 2H), 7.29 – 7.22 (m, 1H), 7.12 (s, 1H), 3.59 (s, 2H), 2.42 (s, 3H).

Figure A.2.6 – $^1$H NMR of 2-(2-methyl-5-phenylthiophen-3-yl) acetic acid (500 MHz, CDCl$_3$).

$^1$H NMR (400 MHz, CDCl$_3$): 5.75 – 7.52 (m, 2H), 7.42 – 7.34 (m, 2H), 7.31 – 7.25 (m, 1H), 7.11 (s, 1H), 5.70 (s, J = 8.8 Hz, 2H), 3.54 (s, 2H), 2.44 (s, 3H).

Figure A.2.7 – $^1$H NMR of 2,2-(2-methyl-5-phenylthiophen-3-yl) acetamide (400 MHz, CDCl$_3$).
Figure A.2.8 – $^{13}$C NMR of 2-(2-methyl-5-phenylthiophen-3-yl) acetamide (101 MHz, CDCl$_3$).

$^1$H NMR (500 MHz, DMSO-d$_6$) δ 11.08 (s, 1H), 7.59 – 7.51 (m, 2H), 7.47 – 7.36 (m, 4H), 7.33 – 7.27 (m, 2H), 7.13 – 7.06 (m, 2H), 6.92 – 6.86 (m, 3H), 3.77 (s, 3H), 2.21 (s, 3H), 1.75 (s, 3H).

Figure A.2.8 – $^1$H NMR of 3-(1,2-dimethyl-1H-indol-3-yl)-4-(2-methyl-5-phenylthiophen-3-yl)-1H-pyrrole-2,5-dion (400 MHz, DMSO-d$_6$).
Figure A.2.9 - $^1$H NMR of 1-methyl-3-(2-methyl-1H-indol-3-yl)-4-(2-methyl-5-phenylthiophen-3-yl)-1H-pyrrole-2,5-dione (400 MHz, DMSO-$d_6$).

$^1$C NMR (101 MHz, DMSO-$d_6$): 171.95, 171.05, 139.89, 139.61, 138.89, 138.04, 134.83, 133.70, 129.96, 129.64, 129.60, 128.80, 125.43, 125.39, 121.66, 120.00, 119.78, 111.35, 103.04, 24.49, 14.94, 13.53.

Figure A.2.10 – $^{13}$C NMR of 1-methyl-3-(2-methyl-1H-indol-3-yl)-4-(2-methyl-5-phenylthiophen-3-yl)-1H-pyrrole-2,5-dione (101 MHz, DMSO-$d_6$).
Figure A.2.11 – $^1$H NMR of 3-(2-methyl-1H-indol-3-yl)-4-(2-methyl-5-phenylthiophen-3-yl)furan-2,5-dione (400 MHz, DMSO-$d_6$).

$^{13}$C NMR (101 MHz, DMSO-$d_6$) 166.03, 165.87, 141.23, 140.49, 140.38, 136.64, 136.09, 133.45, 130.64, 129.71, 128.54, 128.39, 128.37, 125.50, 124.87, 122.14, 121.47, 119.89, 111.62, 102.52, 14.96, 13.81.

Figure A.2.12 – $^{13}$C NMR of 3-(2-methyl-1H-indol-3-yl)-4-(2-methyl-5-phenylthiophen-3-yl)furan-2,5-dione (101 MHz, DMSO-$d_6$).
**Figure A.2.13** – $^1$H NMR of 3-(2-methyl-1H-indol-3-yl)-4-(2-methyl-5-phenylthiophen-3-yl)-1H-pyrrole-2,5-dione (400 MHz, DMSO-d$_6$).

$^1$H NMR (400 MHz, DMSO) $\delta$ 11.55 (s, 1H), 11.06 (s, 1H), 7.55 (d, $J = 7.5$ Hz, 2H), 7.47 – 7.35 (m, 3H), 7.35 – 7.24 (m, 2H), 7.12 – 6.96 (m, 2H), 6.84 (t, $J = 7.5$ Hz, 1H), 2.18 (s, 3H), 1.75 (s, 3H).

**Figure A.2.14** – $^{13}$C NMR of 3-(2-methyl-1H-indol-3-yl)-4-(2-methyl-5-phenylthiophen-3-yl)-1H-pyrrole-2,5-dione (101 MHz, DMSO-d$_6$).

$^{13}$C NMR (101 MHz, DMSO) $\delta$ 172.01, 171.73, 139.28, 139.01, 138.02, 135.53, 134.91, 133.25, 130.23, 129.18, 129.14, 127.51, 126.39, 125.03, 124.94, 121.11, 119.47, 119.25, 110.81, 102.54, 14.42, 13.03.
**Figure A.2.15** – $^1$H NMR of 3-bromo-5-(4-methoxyphenyl)-2-methylthiophene (400 MHz, CDCl$_3$).

$^1$H NMR (400 MHz, DMSO-$d_6$): 6.11–6.57 (s, 1H), 7.09 – 7.17 (m, 2H), 7.33 – 7.36 (m, 2H), 7.65 (m, 4H), 6.88 – 6.91 (m, 1H), 3.78 (s, 3H), 3.05 (s, 3H), 2.17 (s, 3H), 1.74 (s, 3H).

**Figure A.2.16** – $^1$H NMR of 3-(5-(4-methoxyphenyl)-2-methylthiophen-3-yl)-1-methyl-4-(2-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (400 MHz, DMSO-$d_6$).
Figure A.2.17 – $^{13}$C NMR of 3-(5-(4-methoxyphenyl)-2-methylthiophen-3-yl)-1-methyl-4-(2-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (101 MHz, DMSO-d$_6$).

$^1$H NMR (400 MHz, DMSO-d$_6$) δ 11.54 (s, 1H), 11.04 (s, 1H), 7.51 – 7.42 (m, 2H), 7.33 – 7.25 (m, 2H), 7.08 – 6.93 (m, 4H), 6.88 – 6.80 (m, 1H), 5.78 (s, 3H), 2.17 (s, 3H), 1.74 (s, 3H).

Figure A.2.18 – $^1$H NMR of 3-(5-(4-methoxyphenyl)-2-methylthiophen-3-yl)-4-(2-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (400 MHz, DMSO-d$_6$).
Figure A.2.19 – $^{13}$C NMR of 3-(5-(4-methoxyphenyl)-2-methylthiophen-3-yl)-4-(2-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (101 MHz, DMSO-d$_6$).

$^1$H NMR (500 MHz, CDC$_3$) δ 7.39 (d, $J$ = 8.6 Hz, 2H), 6.94 (s, 1H), 6.73 (m, 2H), 2.99 (s, 6H), 2.39 (s, 3H).

Figure A.2.20 – $^1$H NMR of 4-(4-bromo-5-methylthiophen-2-yl)-N,N-dimethylaniline (500 MHz, CDC$_3$).
**Figure A.2.21** – $^1$H NMR of 3-(5-(4-(dimethylamino)phenyl)-2-methylthiophen-3-yl)-1-methyl-4-(2-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (500 MHz, DMSO-d$_6$).

$^1$H NMR (500 MHz, DMSO) δ 11.56 (s, 1H), 7.35 (d, $J$ = 8.8 Hz, 2H), 7.30 (d, $J$ = 8.9 Hz, 1H), 7.17 (s, 1H), 7.08 (d, $J$ = 7.9 Hz, 1H), 7.02 (t, $J$ = 7.7 Hz, 1H), 6.84 (d, $J$ = 7.5 Hz, 1H), 6.74 (d, $J$ = 8.8 Hz, 2H), 3.05 (s, 3H), 2.93 (s, 6H), 2.17 (s, 3H), 1.72 (s, 3H).

**Figure A.2.22** – $^{13}$C NMR of 3-(5-(4-(dimethylamino)phenyl)-2-methylthiophen-3-yl)-1-methyl-4-(2-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (101 MHz, DMSO-d$_6$).
\[ \text{H NMR (500 MHz, DMSO)} \delta 11.53 (s, 1H), 7.29 (d, J = 8.0 Hz, 1H), 7.07 - 6.98 (m, 2H), 6.86 (t, J = 7.5 Hz, 1H), 6.74 (s, 1H), 3.02 (s, 3H), 2.37 (s, 3H), 2.11 (s, 3H), 1.86 (s, 3H). \]

\[ \text{\textit{Figure A.2.23} – } \text{\textsuperscript{1}H NMR of 3-(2,5-dimethylthiophen-3-yl)-1-methyl-4-(2-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (500 MHz, DMSO-\textsubscript{d6}).} \]

\[ \text{\textsuperscript{13}C NMR (101 MHz, DMSO)} \delta 171.41, 171.13, 138.43, 137.66, 135.98, 135.91, 134.20, 130.48, 128.00, 127.22, 126.88, 121.56, 119.91, 119.80, 111.27, 103.12, 24.44, 15.27, 14.73, 13.42. \]

\[ \text{\textit{Figure A.2.24} – } \text{\textsuperscript{13}C NMR of 3-(2,5-dimethylthiophen-3-yl)-1-methyl-4-(2-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (101 MHz, DMSO-\textsubscript{d6}).} \]
$^1$H NMR (400 MHz, DMSO) δ 11.27 (s, 2H), 10.85 (s, 1H), 7.22 (d, $J$ = 8.0 Hz, 2H), 7.01 (d, $J$ = 7.8 Hz, 2H), 6.38 – 6.32 (m, 2H), 6.75 (t, $J$ = 7.3 Hz, 2H), 1.97 (s, 6H).

Figure A.2.25 – $^1$H NMR of 3,4-bis(2-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (400 MHz, DMSO-d$_6$).

$^1$C NMR (101 MHz, DMSO) δ 172.88, 135.90, 132.36, 127.11, 121.19, 119.76, 119.56, 111.05, 103.74, 13.45.

Figure A.2.26 – $^{13}$C NMR of 3,4-bis(2-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (101 MHz, DMSO-d$_6$).
$^1$H NMR (400 MHz, DMSO-d$_6$): δ 11.05 (s, 1H), 10.90 (s, 1H), 8.22 (s, 1H), 7.42 (d, $J = 7.8$ Hz, 1H), 7.20 (dd, $J = 11.2$, 8.2 Hz, 2H), 7.05–8.83 (m, 4H), 6.71–6.63 (m, 1H), 4.46 (d, $J = 30.1$ Hz, 2H), 2.03 (s, 3H), 1.77 (s, 3H).

Figure A.2.27 – $^1$H NMR of 3,4-bis(2-methyl-1H-indol-3-yl)-1H-pyrrol-2(5H)-one (400 MHz, DMSO-d$_6$).

$^1$H NMR (500 MHz, CDCl$_3$): δ 9.83 (s, 1H), 7.62 (d, $J = 3.7$ Hz, 1H), 6.91 (dd, $J = 3.6, 0.7$ Hz, 1H), 2.80 (s, 4H).

Figure A.2.28 – $^1$H NMR of 5-methylthiophene-2-carbaldehyde (500 MHz, CDCl$_3$).
**Figure A.2.39** – $^1$H NMR of (E)-3-(4-bromo-5-methylthiophen-2-yl)acrylic acid (400 MHz, DMSO-$d_6$).

$^1$H NMR (400 MHz, DMSO) $\delta$ 12.44 (s, 1H), 7.62 (d, $J = 15.7$ Hz, 1H), 7.46 (s, 1H), 6.13 (d, $J = 15.8$ Hz, 1H), 2.38 (s, 3H).

**Figure A.2.30** – $^{13}$C NMR of (E)-3-(4-bromo-5-methylthiophen-2-yl)acrylic acid (101 MHz, DMSO-$d_6$).

$^{13}$C NMR (101 MHz, DMSO) $\delta$ 167.55, 138.00, 136.79, 136.13, 133.69, 118.23, 110.35, 15.33.
$^1\text{H NMR}$ (400 MHz, DMSO) $\delta$ 6.74 (s, 1H), 4.52 (t, $J = 5.1$ Hz, 1H), 3.46 – 3.38 (m, 2H), 2.75 (t, $J = 7.6$ Hz, 2H), 2.30 (s, 3H), 1.78 – 1.65 (m, 2H).

Figure A.2.31 – $^1\text{H NMR}$ of 3-(4-bromo-5-methylthiophen-2-yl)propan-1-ol (400 MHz, DMSO-d$_6$).

$^{13}\text{C NMR}$ (101 MHz, DMSO) $\delta$ 143.15, 131.50, 126.98, 107.86, 60.03, 34.49, 26.31, 14.69.

Figure A.2.32 – $^{13}\text{C NMR}$ of 3-(4-bromo-5-methylthiophen-2-yl)propan-1-ol (101 MHz, DMSO-d$_6$).
Figure A.2.33 – $^1$H NMR of 3-(4-bromo-5-methylthiophen-2-yl)propyl 4-methylbenzenesulfonate (400 MHz, CDCl$_3$).

$^1$H NMR (400 MHz, DMSO) $\delta$ 6.74 (s, 1H), 2.72 (t, $J$ = 7.5 Hz, 2H), 2.30 (s, 3H), 2.11 (s, 6H), 1.68 (quintet, $J$ = 7.1 Hz, 2H).

Figure A.2.34 – $^1$H NMR of of 3-(4-bromo-5-methylthiophen-2-yl)-N,N-dimethylpropan-1-amine (400 MHz, DMSO-d$_6$).
**Figure A.2.35** – $^{13}$C NMR of 3-(4-bromo-5-methylthiophen-2-yl)-N,N-dimethylpropan-1-amine (101 MHz, DMSO-$d_6$).

$^1$H NMR (400 MHz, DMSO) $\delta$ 11.50 (s, 1H), 10.96 (s, 1H), 7.28 (d, $J = 8.0$ Hz, 1H), 7.06 – 6.95 (m, 2H), 6.82 (t, $J = 7.5$ Hz, 1H), 6.71 (s, 1H), 2.70 (t, $J = 7.1$ Hz, 2H), 2.21 – 2.06 (m, 11H), 1.89 (d, $J = 6.9$ Hz, 3H), 1.68 – 1.59 (m, 2H).

**Figure A.2.36** – $^1$H NMR of 3-(5-(3-(dimethylamino)propyl)-2-methylthiophen-3-yl)-4-(2-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (400 MHz, DMSO-$d_6$).
Figure A.2.37 – $^{13}$C NMR of 3-(5-(3-(dimethylamino)propyl)-2-methylthiophen-3-yl)-4-(2-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (101 MHz, DMSO-d$_6$).

$^1$H NMR (400 MHz, DMSO-d$_6$) δ 8.20 (s, 1H), 7.82 (d, $J = 8.1$ Hz, 1H), 7.44 – 6.96 (m, 6H), 6.85 (s, 1H), 4.90 (t, $J = 6.5$ Hz, 2H), 2.16 (s, 3H), 2.07 – 1.81 (m, 11H), 1.62 (s, 3H).

Figure A.2.38 – $^1$H NMR of 3-(2-methyl-1H-indol-3-yl)-4-(2-methylbenzo[b]thiophen-3-yl)-1H-pyrrole-2,5-dione (400 MHz, DMSO-d$_6$).
Figure A.2.39 – $^1$H NMR of 3-(2-methyl-1H-indol-3-yl)-4-(2-methylbenzo[b]thiophen-3-yl)-1H-pyrrole-2,5-dione (101 MHz, DMSO-$d_6$).
Chapter 3 – NMR spectroscopy

Figure A.3.1 – 1H NMR (400 MHz, CDCl₃) of compound 4-(2-(5-bromo-2-methylthiophen-3-yl)cyclopent-1-en-1-yl)-5-methyl-thiophene-2-carbaldehyde at 25°C.

Figure A.3.2 - 1H NMR (400 MHz, CDCl₃) spectrum of compound 4-(2-(5-bromo-2-methylthiophen-3-yl)cyclopent-1-en-1-yl)-5-methyl-thiophene-2-carbaldehyde at 25°C.
Figure A.3.3 - $^1$H NMR (400 MHz, CDCl$_3$) spectrum of compound 3.1-o at 25°C.

Figure A.3.4 - $^{13}$C NMR (100 MHz, CDCl$_3$) spectrum of compound 3.1-o at 25°C.
Figure A.3.5 - $^1$H NMR spectrum (400 MHz, CD$_3$CO$_2$D) for a solution (5 × 10$^{-3}$ M) of 3.1-o at 25°C.

Figure A.3.6 - $^1$H NMR spectra (400 MHz, CD$_3$CO$_2$D) for a solution (5 × 10$^{-3}$ M) of 3.1-c (photostationary state, contains 97% of ring closed isomer 3.1-c) when 3.1-o was irradiated with 365 nm light for 30 min.